READY, SET, GO!
GETTING STARTED WITH RNASCOPE®

Presented by:
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May 12th, 2015

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Advanced Cell Diagnostics

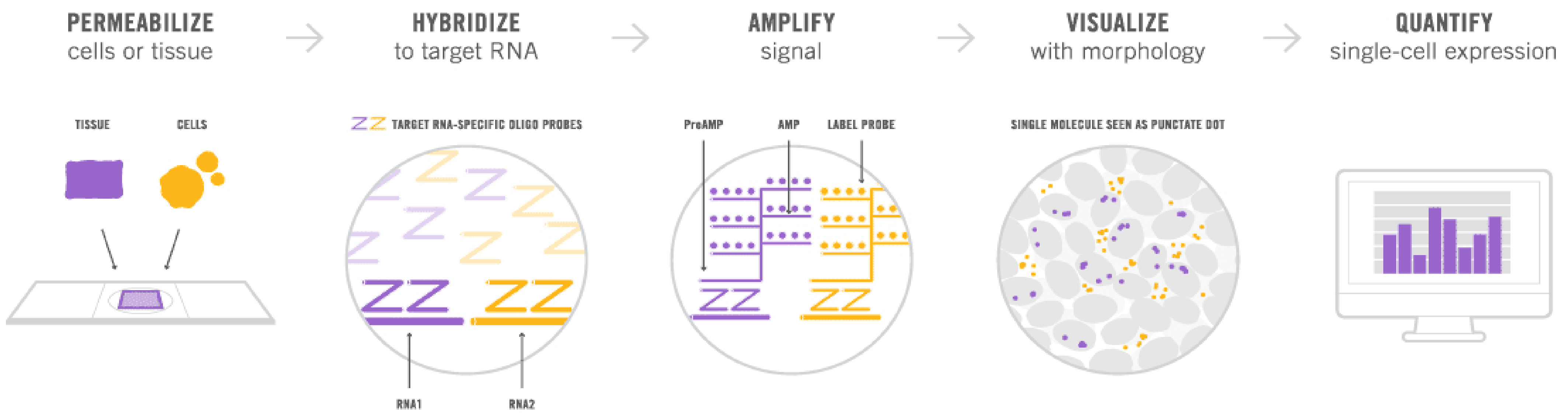
TOPICS

- How Does RNAscope® Work?
- Getting Started with RNAscope® in your Laboratory
- Tips and Tricks on Running the Assay
- Frequently Asked Questions
- Time for Q&A



RNASCOPE® OVERVIEW

RNASCOPE ® WORKFLOW



A BREAKTHROUGH PLATFORM

UNIQUE
Probe Design

SIGNAL
Amplification + Background Suppression

SINGLE
Molecule Detection in **Single Cells**

ANY
Genome, Gene or Tissue

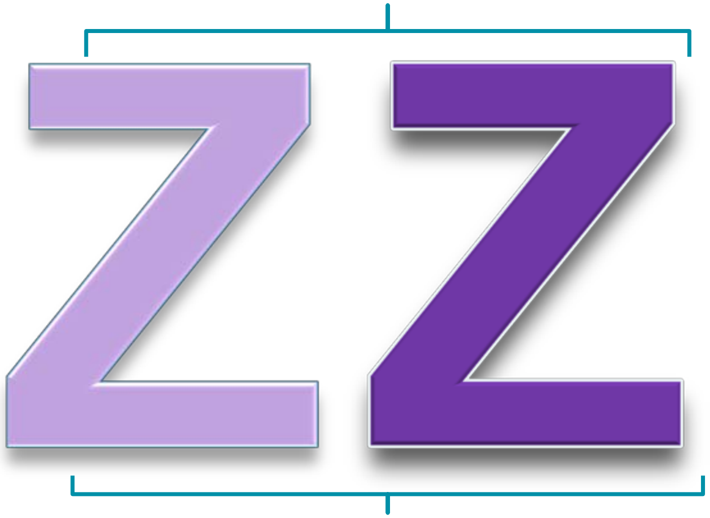
RNASCOPE® TECHNOLOGY: ZZ PROBE DESIGN

Preamplifer binding site: 28-bases

Upper region:
14-base tail sequence

Spacer

Lower region:
~8-25-base sequence



Target-specific binding site: ~50-base region

Target Probe Set:
Pool of 20 ZZ Oligo Pairs

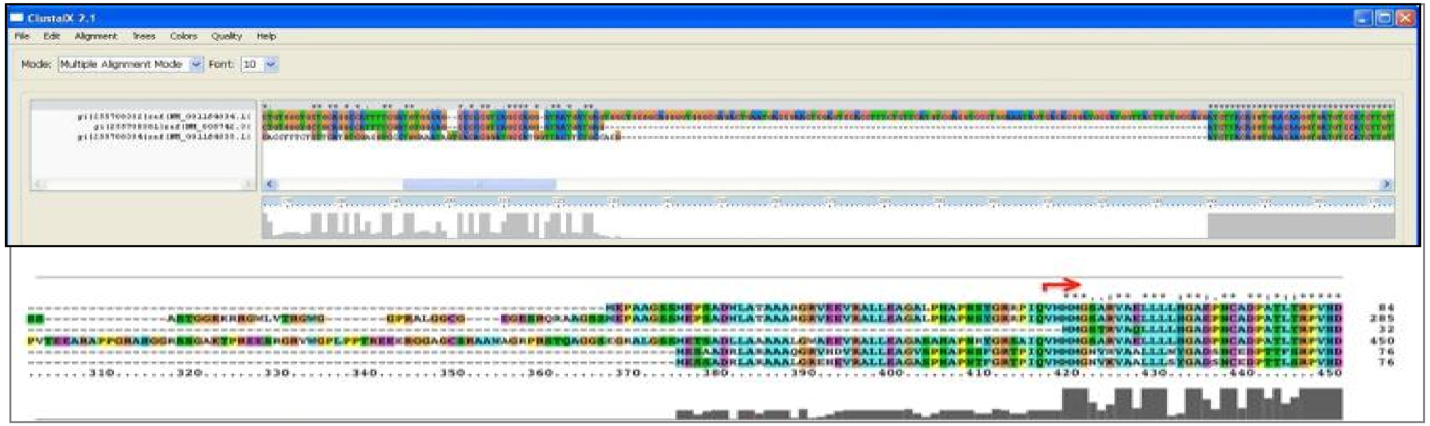


RNA Target Region
1KB
20 pairs of ~50 bases

mRNA transcript detection: Highly specific & robust signal amplification

PROBE DESIGN OVERVIEW

Customer
Gene-of-interest
Accession No.
(e.g.,ENSG00000097007)



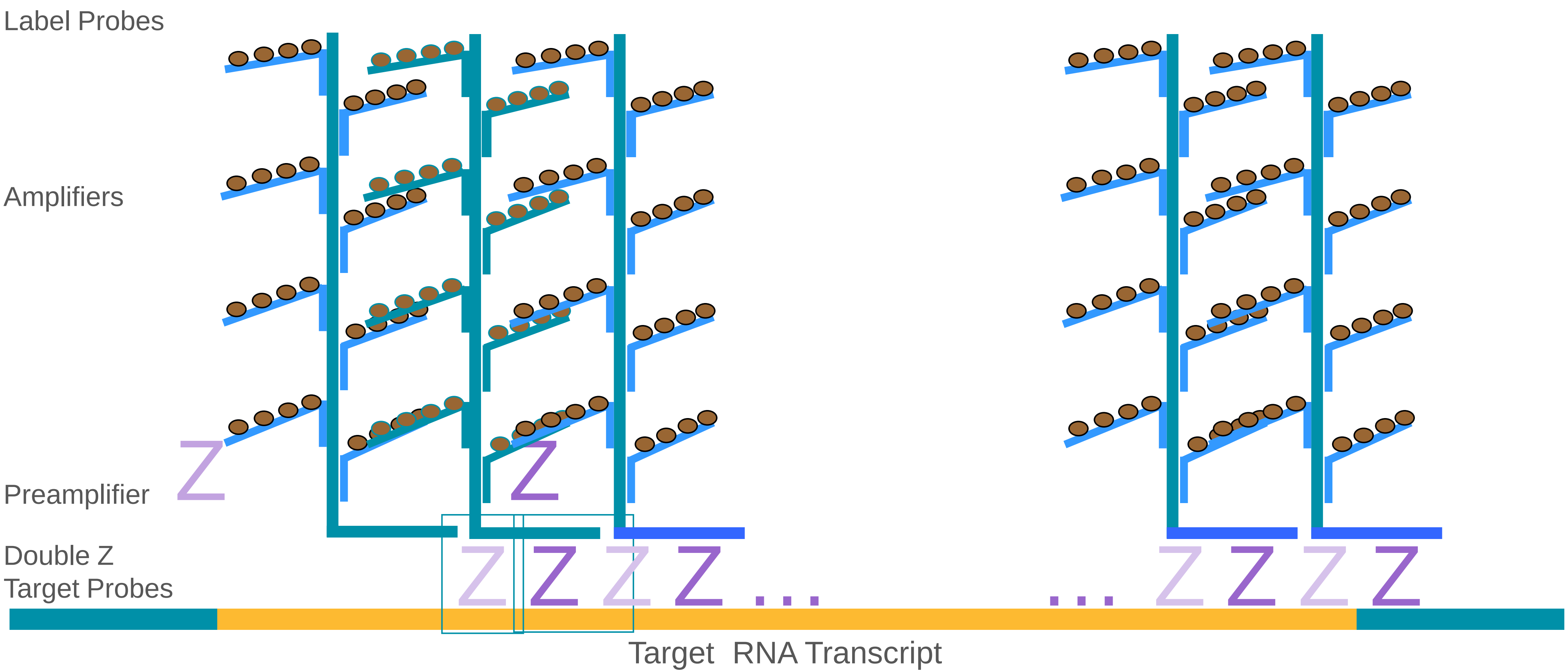
ACD Alignment Algorithm:
Identify Low Homology, Avg. GC Region
(~ 1,000 nucleotides)

Standard Probe Configuration: 20 Z-oligo pairs = 1,000 nt



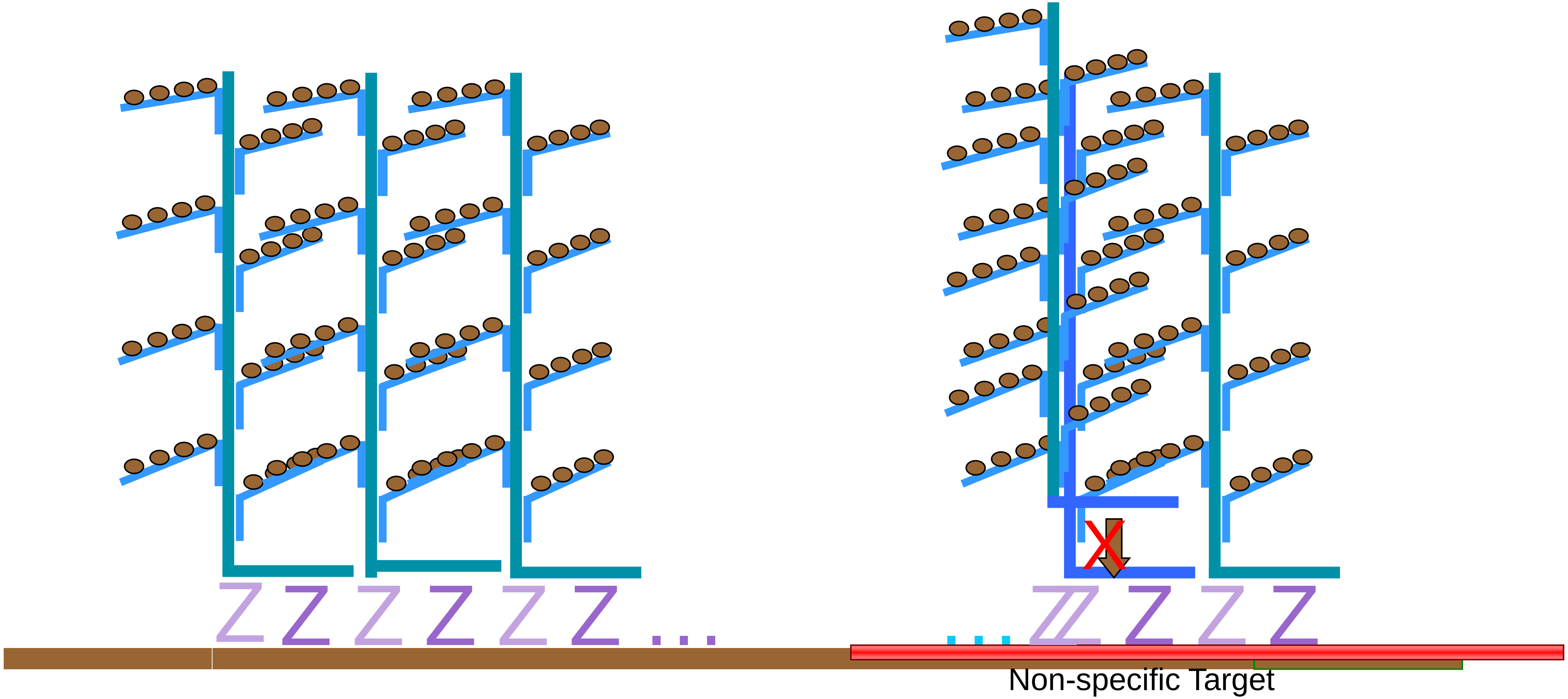
HOW DOES RNASCOPE ® WORK?

SIGNAL AMPLIFICATION



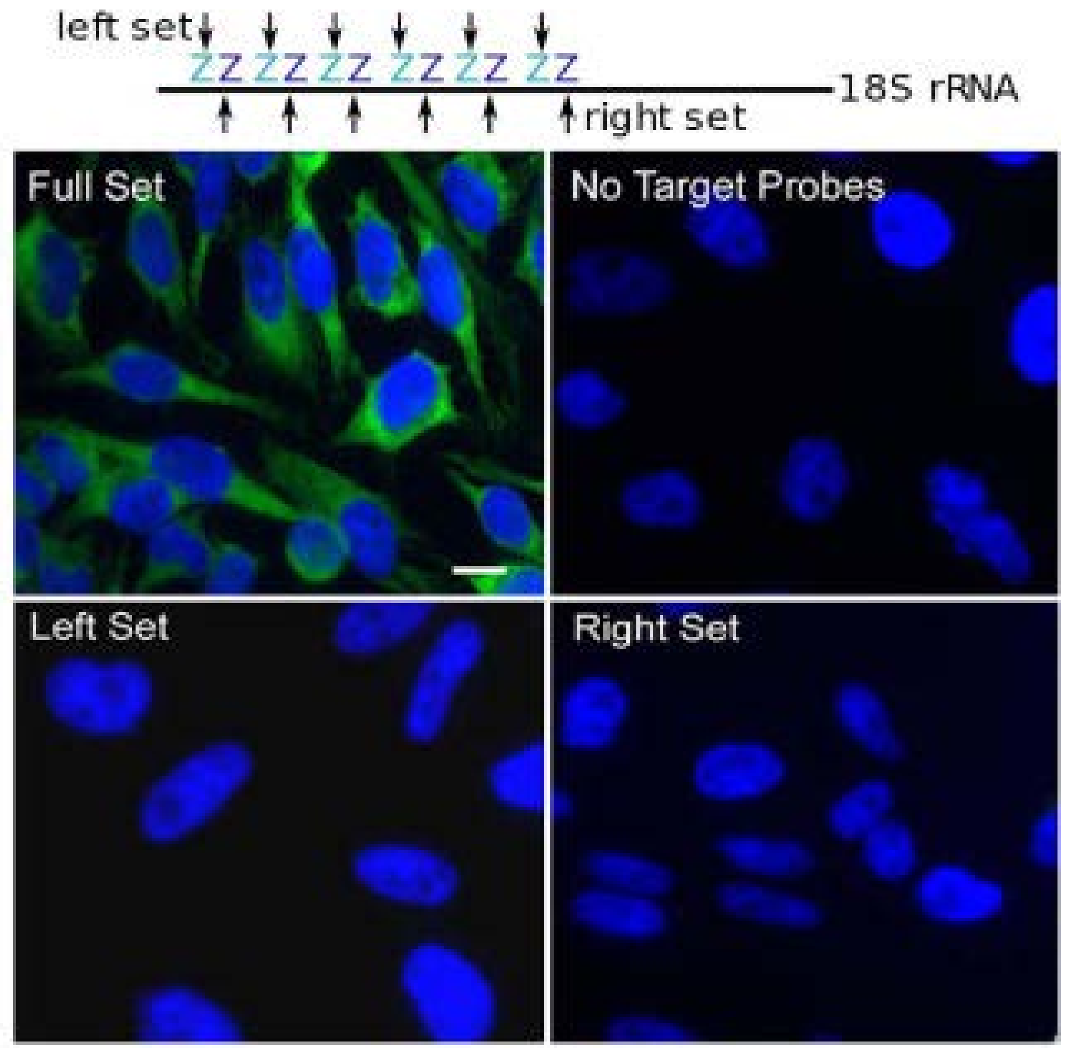
HOW DOES RNASCOPE ® WORK?

SIGNAL AMPLIFICATION



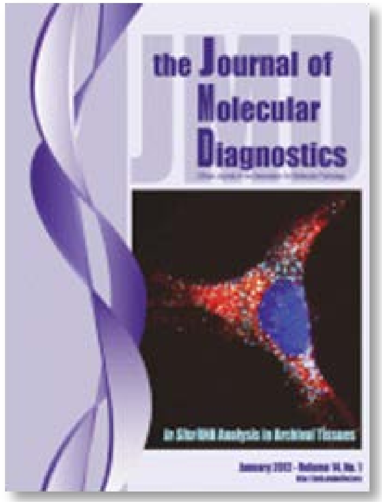
20 Z pairs x 20 Amplifiers x 20 Labels
8000 labelled molecules per 1kb region

HOW DOES RNASCOPE ® WORK?

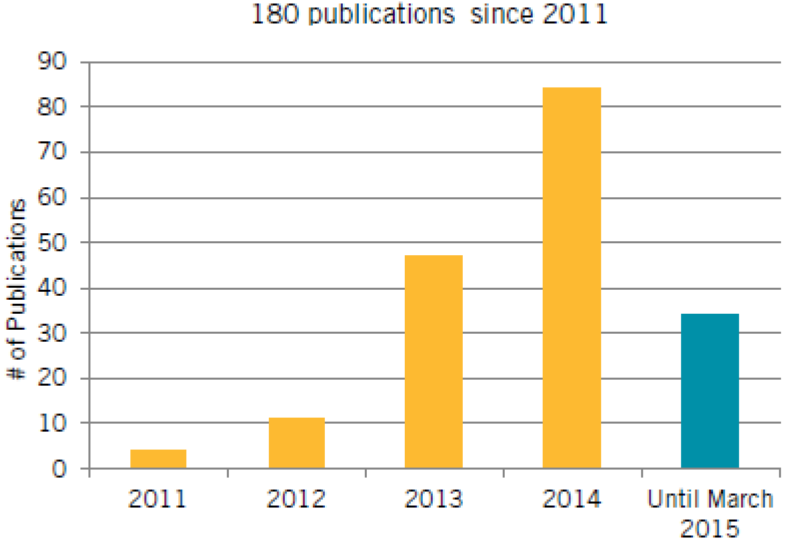


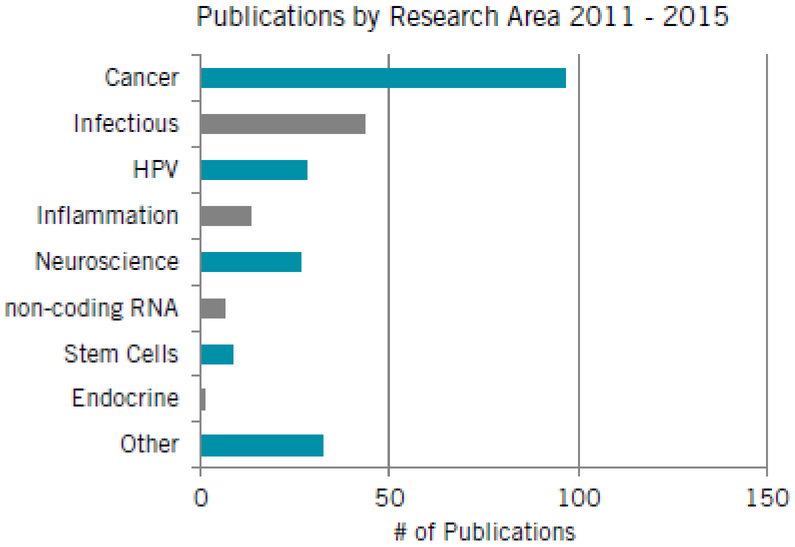
Specific Signal Amplification

RNASCOPE PUBLICATION AND LITERATURE REFERENCES



Wang, F. et al.2012





The New England Journal of Medicine

nature

Cell

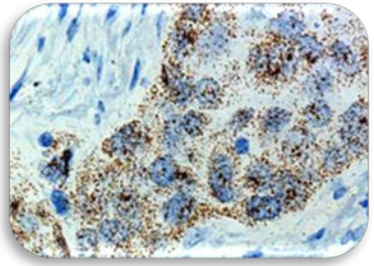
Science AAAS

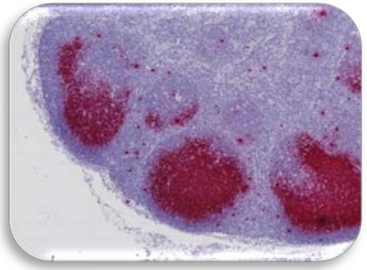
Visit www. acdbio.com and download a publication of your interest

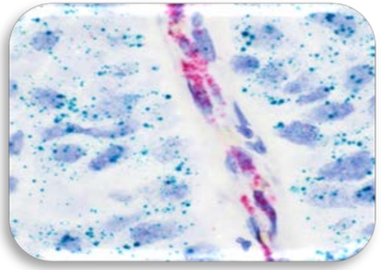


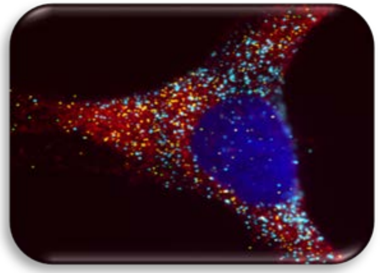
RNASCOPE ® WORKFLOW

RNASCOPE® ASSAY SELECTION





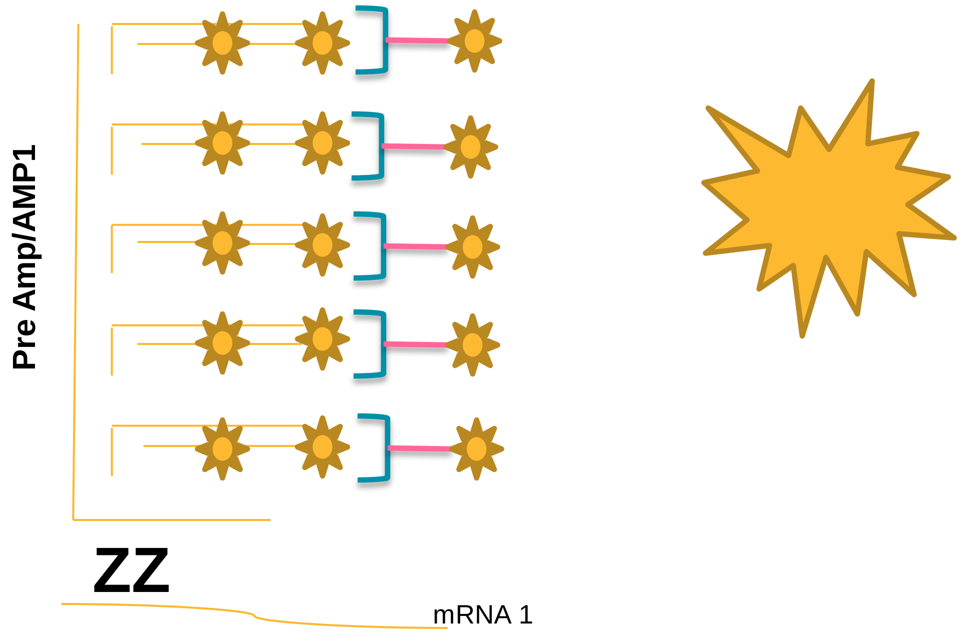




RNAscope Assays	**RNAscope 2.0 HD BROWN**	**RNAscope 2.0 HD RED**	**RNAscope 2-plex**	**RNAscope Multiplex – Fluoroscence**
Assay type	Chromogenic	Chromogenic	Chromogenic	Fluorescent
Dye used	Diaminobenzene (DAB)-HRP	Fast Red -ALP	HRP-Green, Fast Red -ALP	FITC, Cy3, Cy5,
Channel	Channel 1	Channel 1	Channel 1, 2	Channel 1, 2, 3
Probes channel	C1 Probes	C1 Probes	C1, C2 Probes	C1, C2, C3 Probes

RNASCOPE® 2.0 HD AMPLIFICATION SCHEMATIC

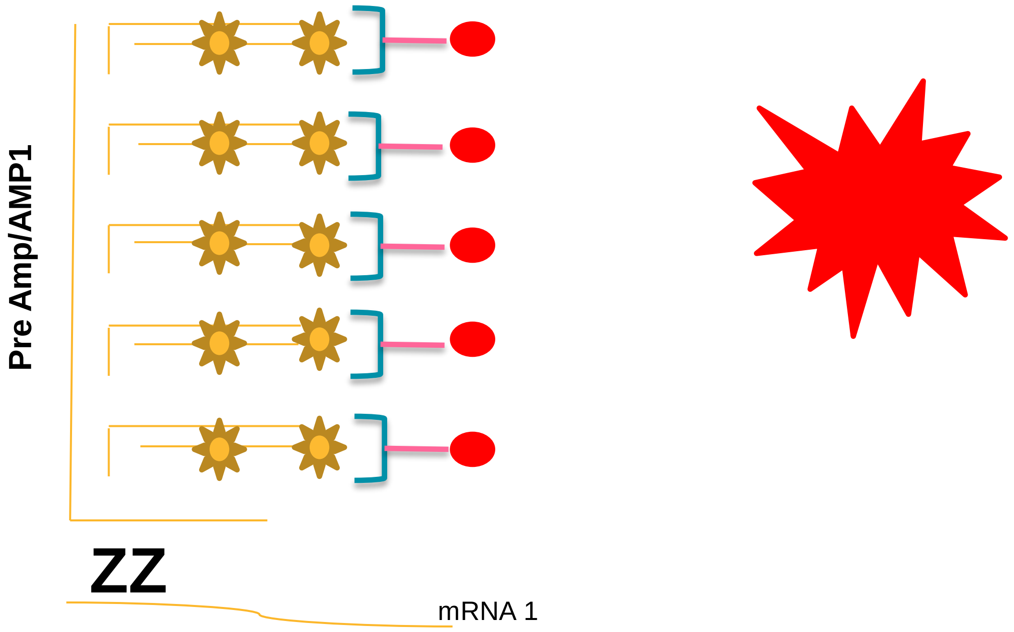
Amplification steps | **Amp6-Brown Label probe** | **Enzyme: HRP Substrate: DAB**



2.0 HD BROWN

Brown dot
HRP/DAB

Amplification steps | **Amp6-Red Label probe** | **Enzyme: ALP Substrate: FastRed**

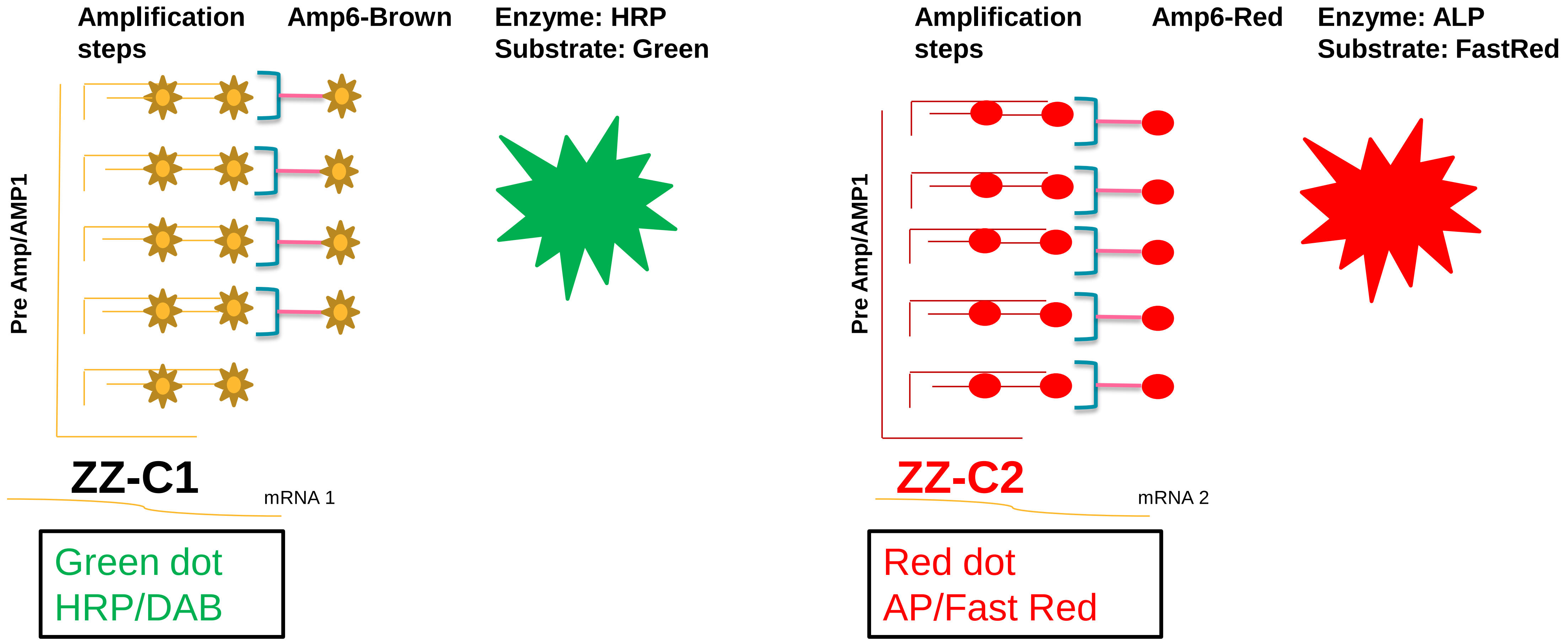


2.0 HD RED

Red dot
AP/Fast Red

TIP : Do not interchange reagents within Brown/Red assays or across similar 2.0 HD Assays
By default 2.0 HD assays require C1 probes that are ready to use, no further dilution is required

RNASCOPE® 2-PLEX AMPLIFICATION SCHEMATIC

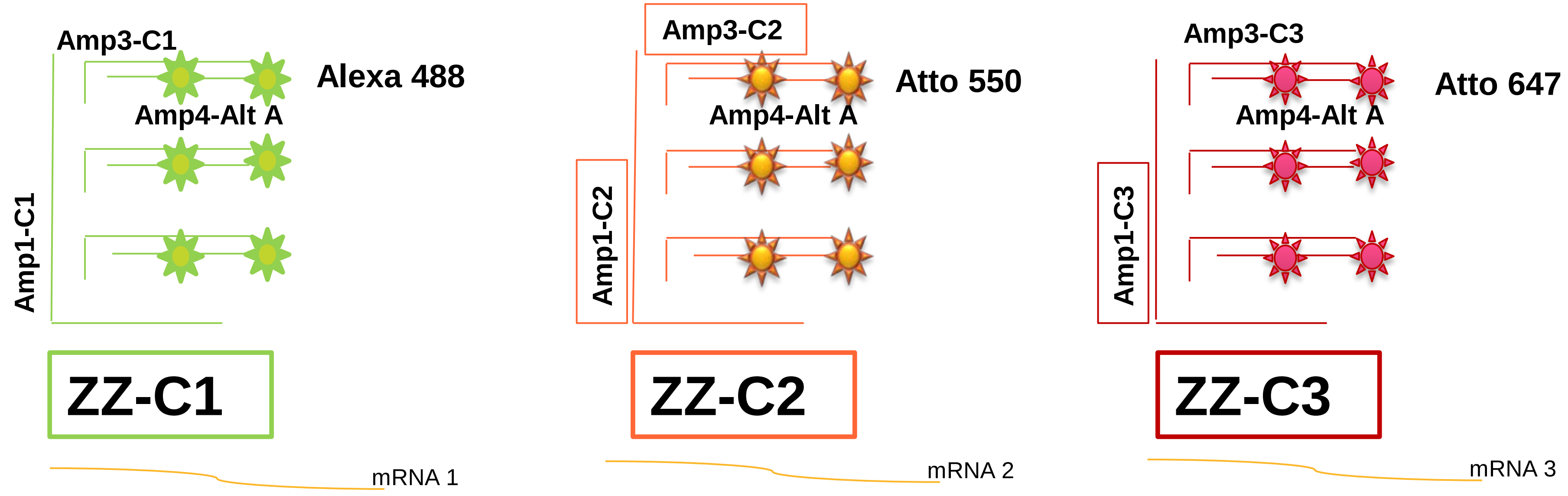


TIP: By default C1 probes are 1X concentration while C2 probes are 50X

To make 2-plex probe mixture at 1X concentration, mix C2 probes 1:50 with C1 probes

To view C2 probes only, use the "blank-probe-C1", as a diluent and mix at a 1:50 dilution

RNASCOPE ® MULTIPLEX FLUORESCENT SCHEMATIC

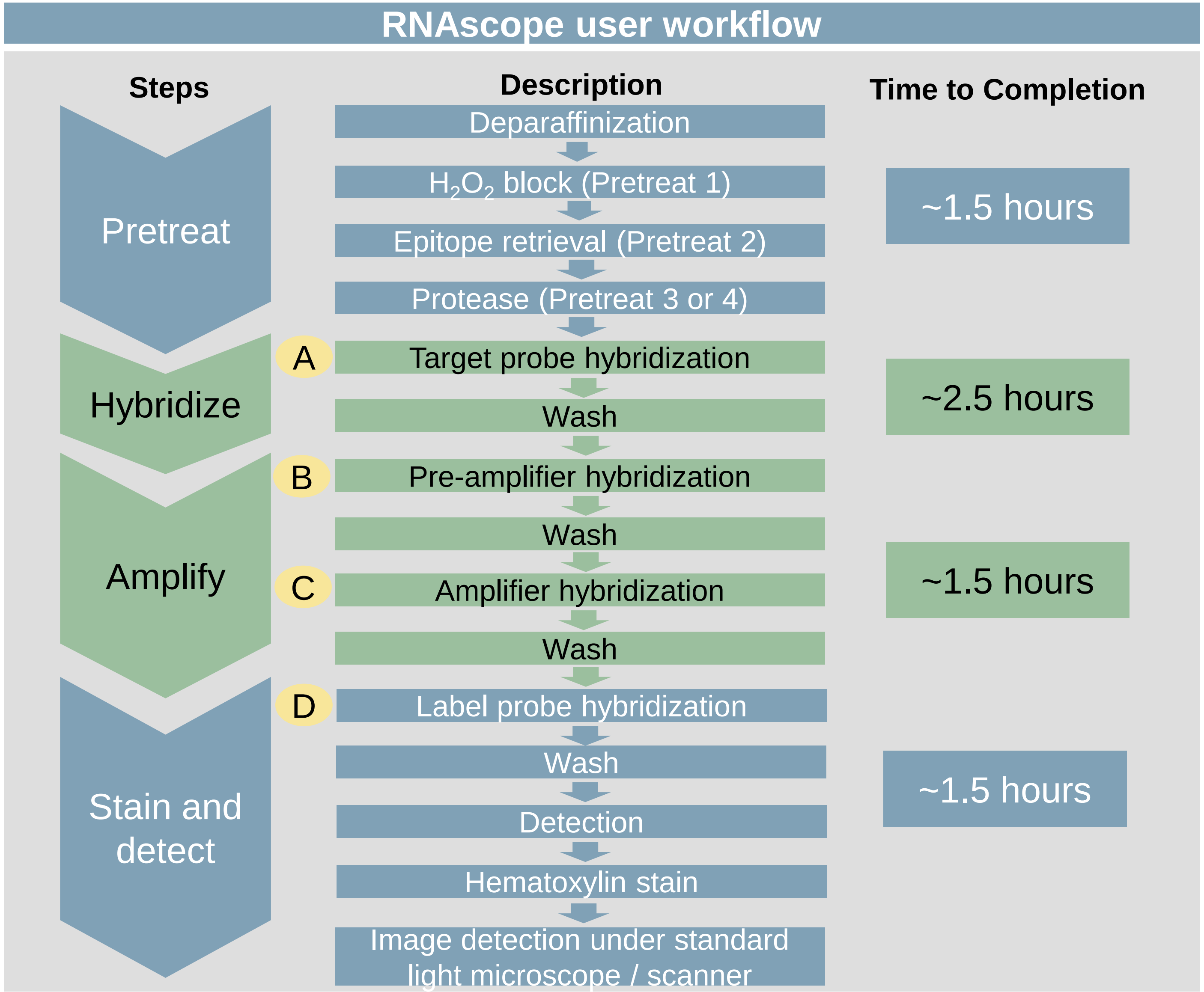


	Alexa-488	Atto-550	Atto-647
Ex/Em	495nm/520nm	555nm/575nm	645nm/670m

Color Module	C1	C2	C3
Amp4 Alt A	GREEN - Alexa 488	ORANGE--Atto 550	FAR RED--Atto 647
Amp4 Alt B	ORANGE--Atto 550	GREEN - Alexa 488	FAR RED--Atto 647
Amp4 Alt C	ORANGE--Atto 550	FAR RED--Atto 647	GREEN - Alexa 488

TIP: By default C1 probes are 1X concentration while C2 and C3 probes are 50X
To make 3-plex probe mixture at 1X concentration, mix C2 and C3 probes 1:50 with C1 probe
If C2 and C3 are all at 50X concentration, use the "blank-probe-C1" as a diluent and mix at a 1:50 dilution

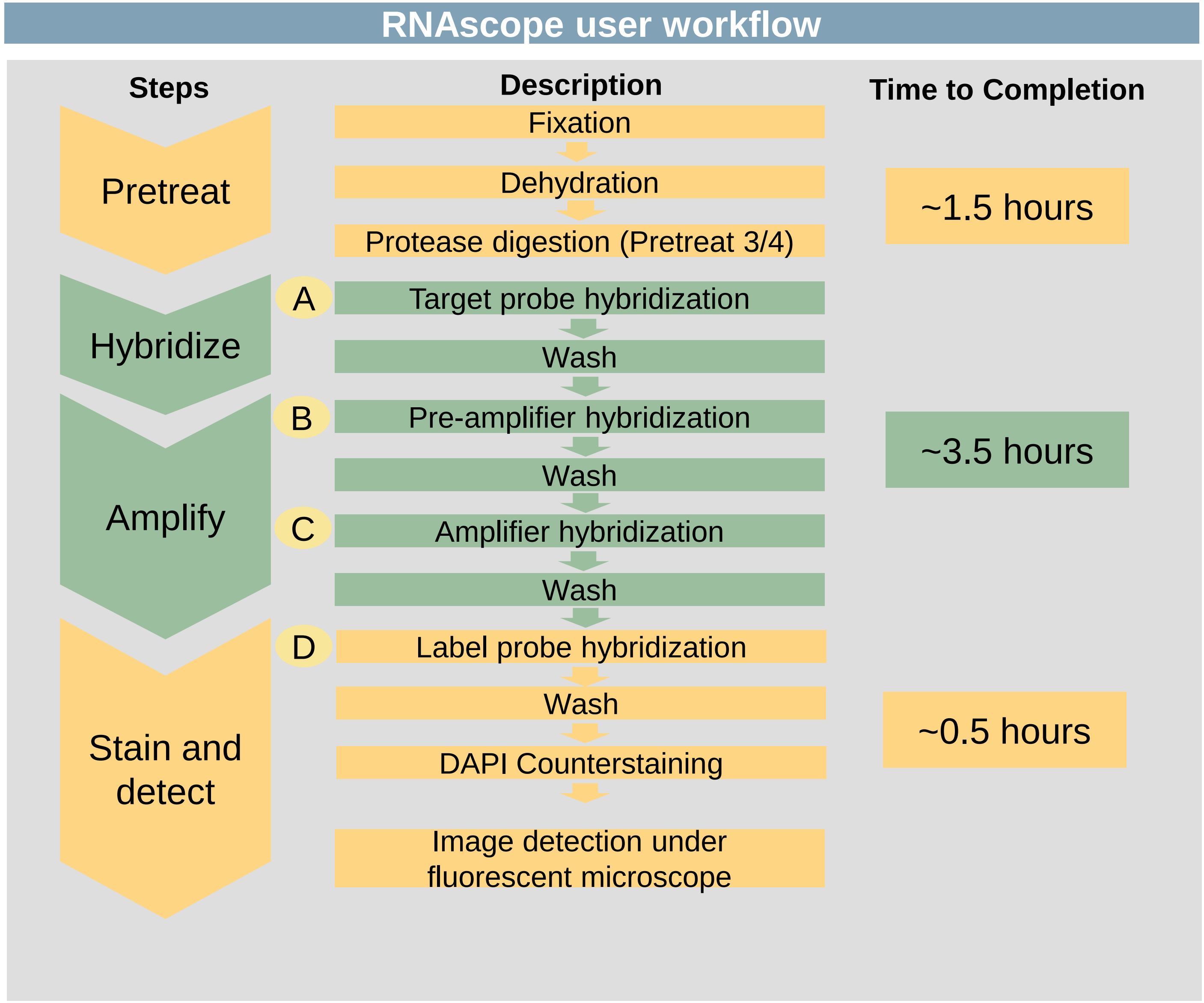
RNASCOPE WORKFLOW: CHROMOGENIC ASSAY



TIP : Detection protocols will vary based on the chromogenic assay used

Download manuals: http://www.acdbio.com/technical-support/downloads

RNASCOPE WORKFLOW: FLUORESCENT ASSAY



TIP : Pretreatment conditions will vary based on sample type

Download manuals: http://www.acdbio.com/technical-support/downloads

ONE DAY OR TWO DAY ASSAY?

ONE DAY ASSAY

Sample preparation

↓

Sample pretreatment

↓

RNAscope assay

TWO DAY ASSAY

Sample preparation

↓ DAY 1

Sample pretreatment

↓ DAY 2

RNAscope assay

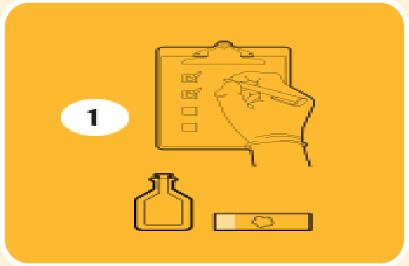
TIP : Review the User Manuals PART 1 and PART 2 for optional stopping points



GETTING STARTED WITH RNASCOPE IN YOUR LAB

GET STARTED BY FOLLOWING 3 EASY STEPS

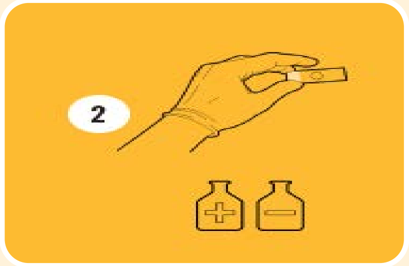
1



Check that all required materials are available

THE CHECKLIST

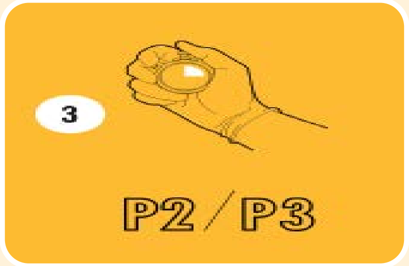
2



Always use control probes and slides

USE CONTROLS

3



Optimize RNAscope assay conditions for your tissues for publication quality data

OPTIMIZE

TIP : Visit www.acdbio.com/go for more information on getting started

THE CHECKLIST: WHAT YOU NEED

- ☑ RNAscope target probes (single-plex and multiplex)
- ☑ Positive and Negative control probe
- ☑ Hot–Plate for pretreatment/ target retrieval step
- ☑ RNAscope reagent kit (Detection Kit, Pretreatment kits & Wash Buffer)
- ☑ HybEZ Hybridization system
- ☑ EZ Batch slide processing system or Tissue-Tek system
- ☑ Ecomount for 2.0 HD Red & 2-plex chromogenic assay
- ☑ RNAscope control slides
- ☑ Immedge hydrophobic barrier pen
- ☑ User supplied reagents (refer to user manual)
- ☑ Read the user manual (Part 1 – Sample Prep & Part 2 – Detection Assay)

TIP : Visit www. acdbio.com/go for more information on getting started.
Checklist is available on the website and in the manual

USING A HOT PLATE

☑ *Hotplate for retrieval/boiling*



TIP : When using a hot plate for pre-treatment step – pay close attention to the TIME and boiling TEMPERATURE

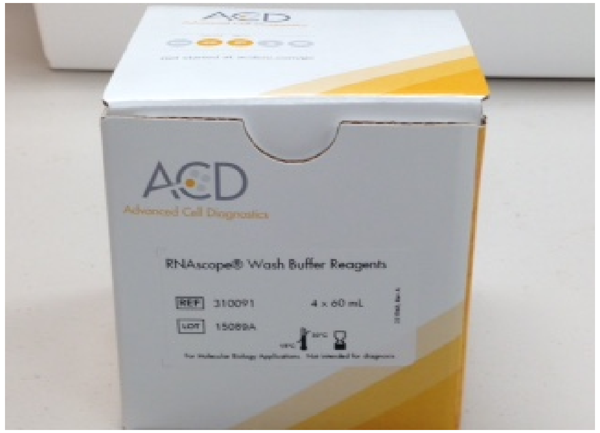
RNASCOPE ® REAGENT KIT CONTENTS

OLD



NEW







Contents of the reagent kit

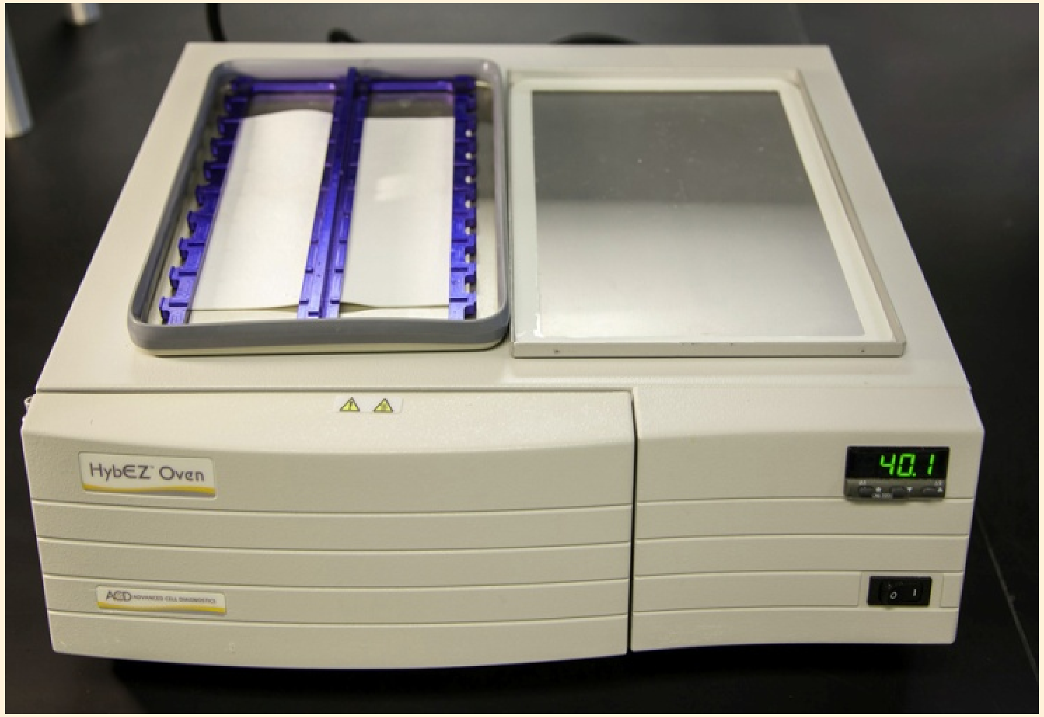
1. *Pretreatment reagents*
2. *RNAscope detection kit*
3. *Wash buffer*

TIP : Warm probes at 40 ºC for 10 minutes before use

TIP :Warm 50x wash buffer at 40 ºC for 20 minutes if you notice a precipitation

USING A HYBEZ HYBRIDIZATION OVEN

☑ ***HyBEZ hybdrization system***





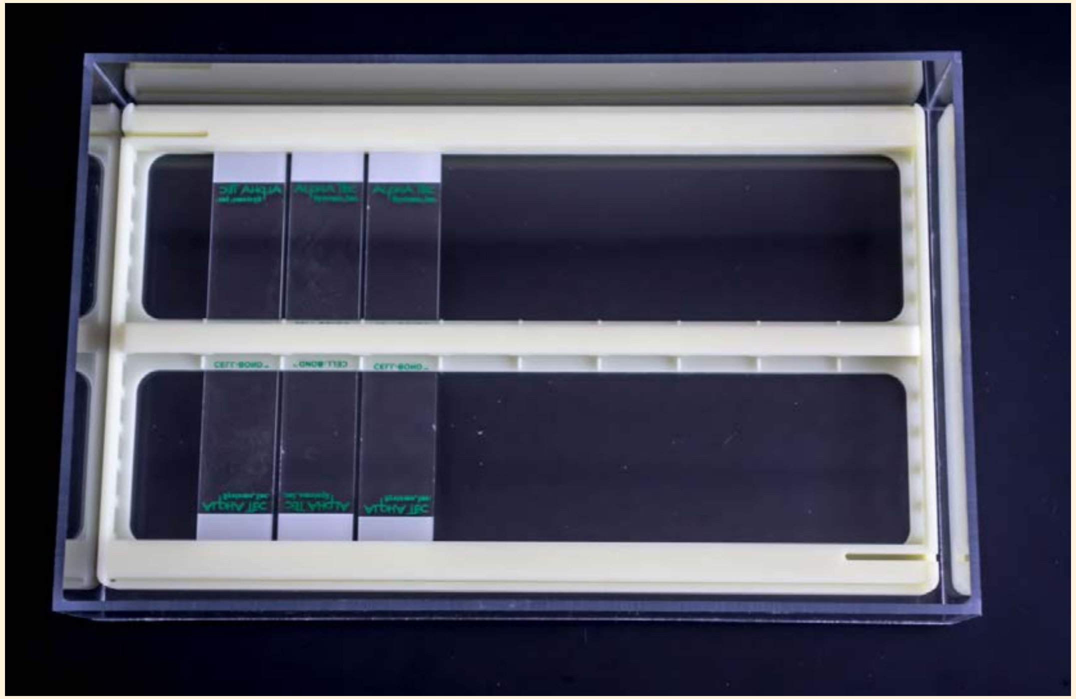
TIP: HybEZ oven is required as it provides both temperature and humidity control, necessary to obtain optimal RNAscope results

ACCESSORIES FOR WASHING STEPS

☑ ***Tissue Tek washing tray***

☑ ***EZ Batch for slide processing***





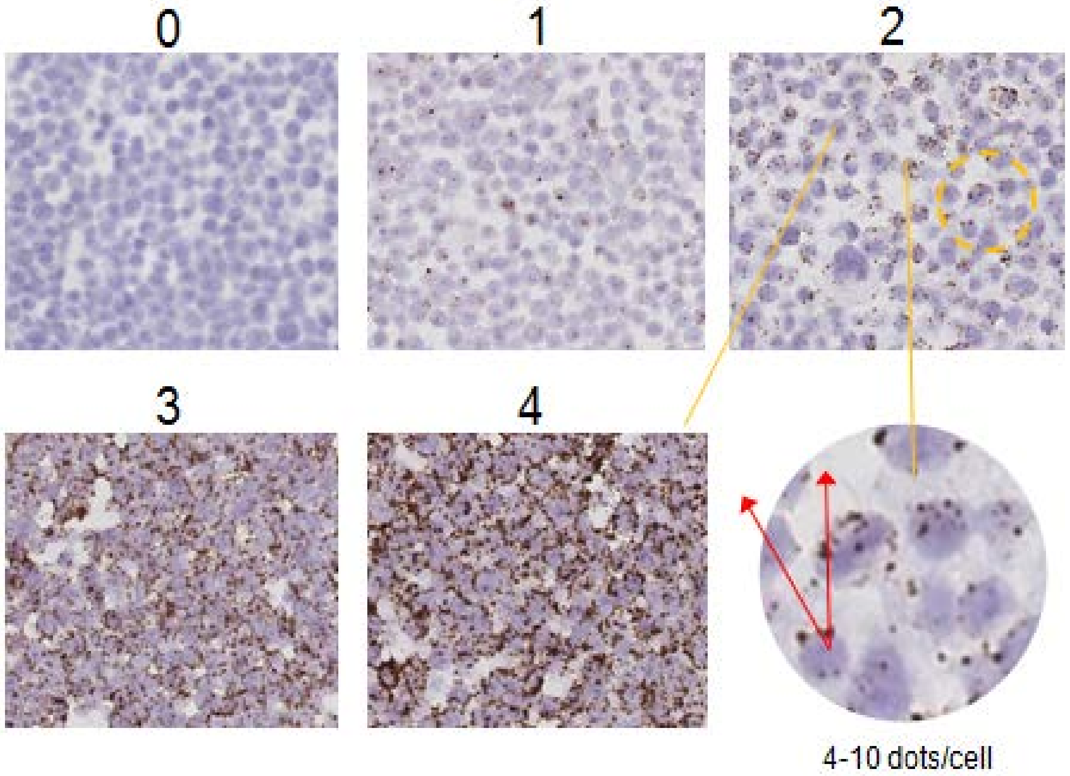
TIP: ACD EZ Batch slide processing tray is easy and convenient for loading multiple slides for washing steps.



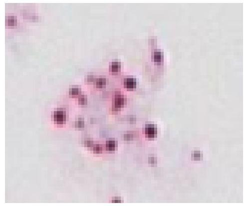
QUALIFY YOUR SAMPLES USING CONTROLS

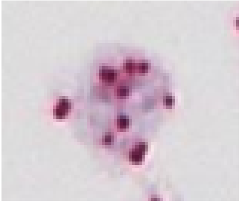
IMAGE ANALYSIS

RNASCOPE® SEMI- QUANTITATIVE SCORING



Score	Criteria
0	No staining or <1 dot/ 10 cells*
1	1-3 dots/cell
2	4-9 dots/cell. None or very few dot clusters
3	10-15 dots/cell and <10% dots are in clusters
4	>15 dots/cell and >10% dots are in clusters


Score = 3


Score = 3

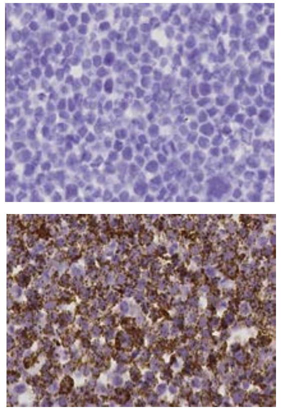
QUALIFY YOUR SAMPLES USING CONTROLS

Control slides e.g. Hela

Negative control probe, e.g. DapB

Positive control probe, e.g. PPIB

QC check
PPIB > 2
DapB < 1

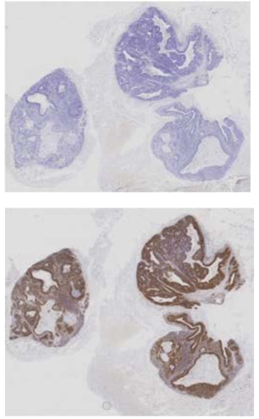


Sample

Negative control probe, e.g. DapB

Positive control probe, e.g. PPIB

QC check
PPIB > 2
DapB < 1



PASS

Run your target probes

FAIL

- *Verify technique*
- *Check RNA quality with new samples*
- *Perform Assay optimization*

Technique check

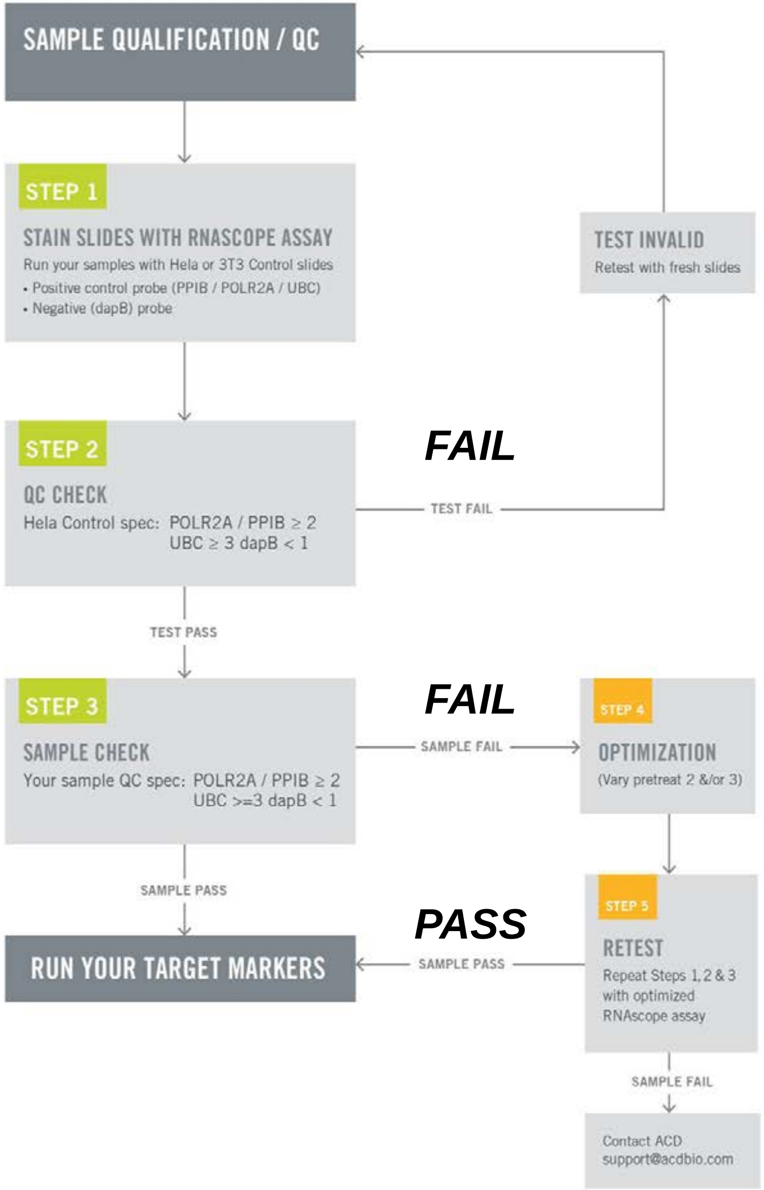
Sample/RNA quality check

TIP : Always start with standard conditions

OPTIMIZE YOUR ASSAY

Technique check

Sample/
RNA quality check



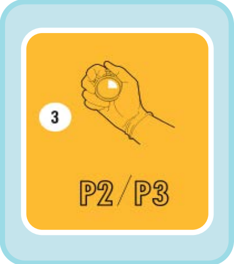
OPTIMIZE YOUR ASSAY →

TIP : Refer to the Troubleshooting Guide



OPTIMIZE YOUR ASSAY

OPTIMIZE YOUR SAMPLE IN 3 EASY STEPS



STEP 1
START WITH STANDARD CONDITIONS

Observe Staining Pattern -
High background, over-digested? = ***underfixed***
No signal/weak signal, under-digested? = ***overfixed***

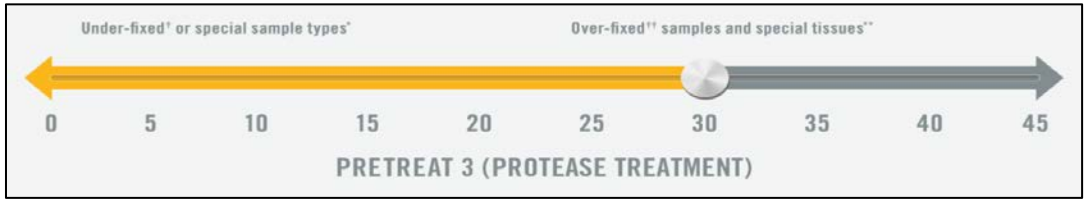
STEP 2

ADJUST PRETREATMENT 2, BOILING TIME



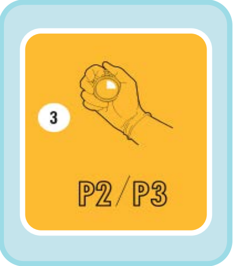
STEP 3

ADJUST PRETREATMENT 3/4, PROTEASE TIME*



TIP: For cultured cells, protease is diluted 1:15 in 1X PBS
* For fresh frozen samples, only protease pretreatment is required and is performed at room temperature

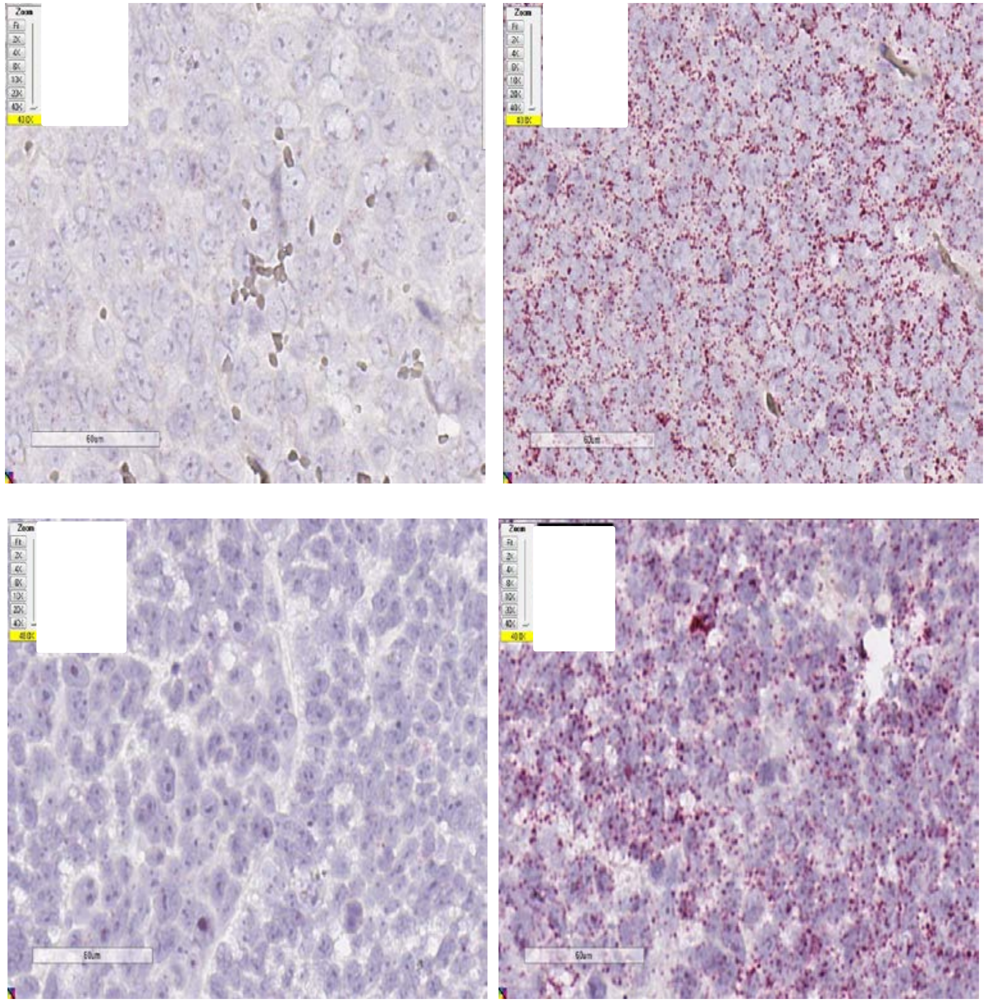
TROUBLESHOOTING: **OVERDIGESTION**



Negative control dapB | *Positive control PPIB*

15 min Pretreat 2 , 30 min Pretreat 3

8 min Pretreat 2 , 30 min Pretreat 3



Sample: FFPE Xenograft

Assay: RNAscope 2.0 HD Red

Issue: Destroyed morphology, ghost nuclei, high nuclear background, weak hematoxylin staining

Optimization: Decrease pretreatment 2 conditions.

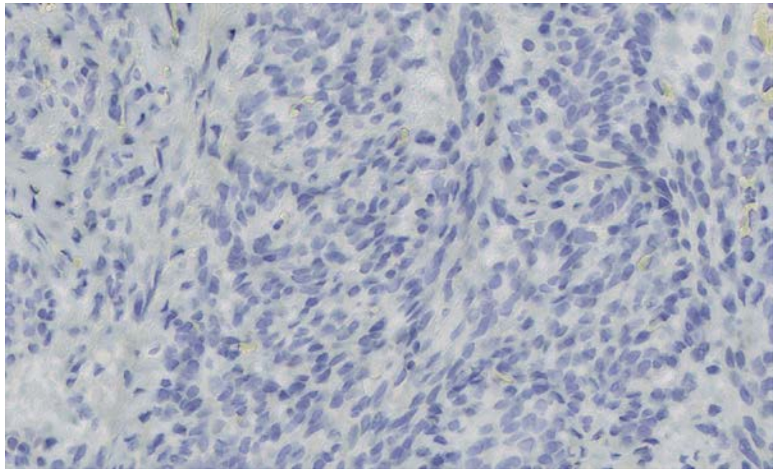
Result: Strong staining for positive controls with no/negligible background, intact morphology, strong hematoxylin staining

TIP: Visit http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/

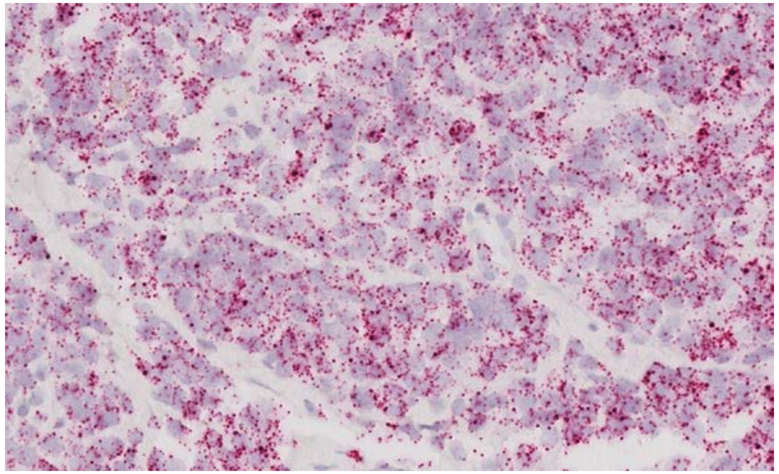
ACD

EXAMPLE OF SUCCESFUL RNASCOPE ® RESULTS

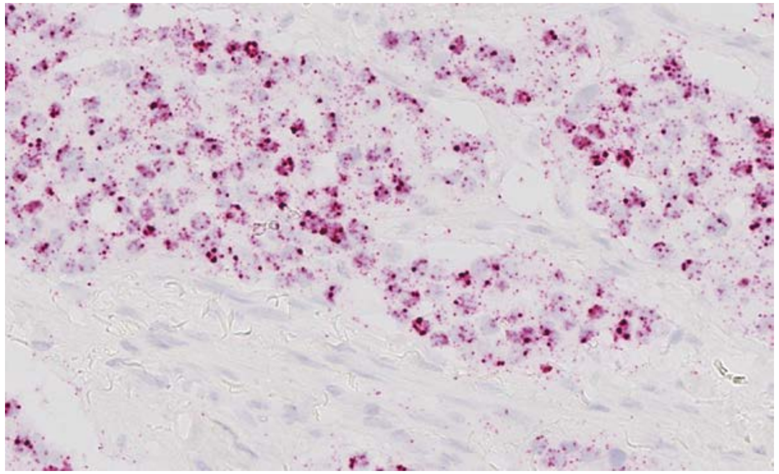
Negative control, DapB



Positive control, PPIB



Target probe

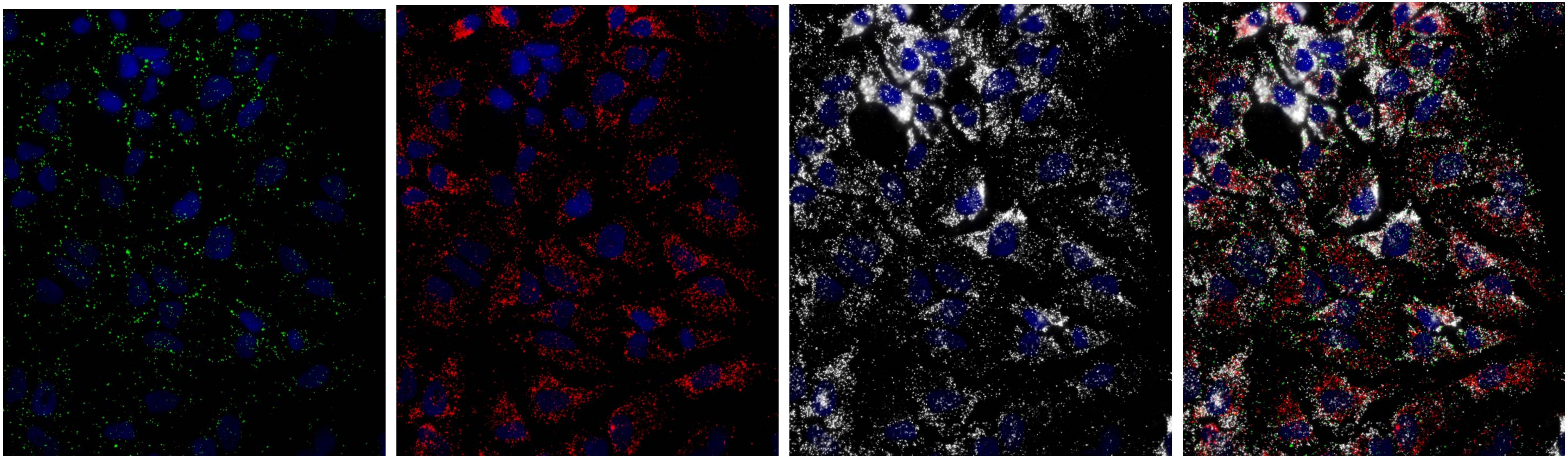


RNAscope 2.0 HD Red Assay

Human breast cancer tissue

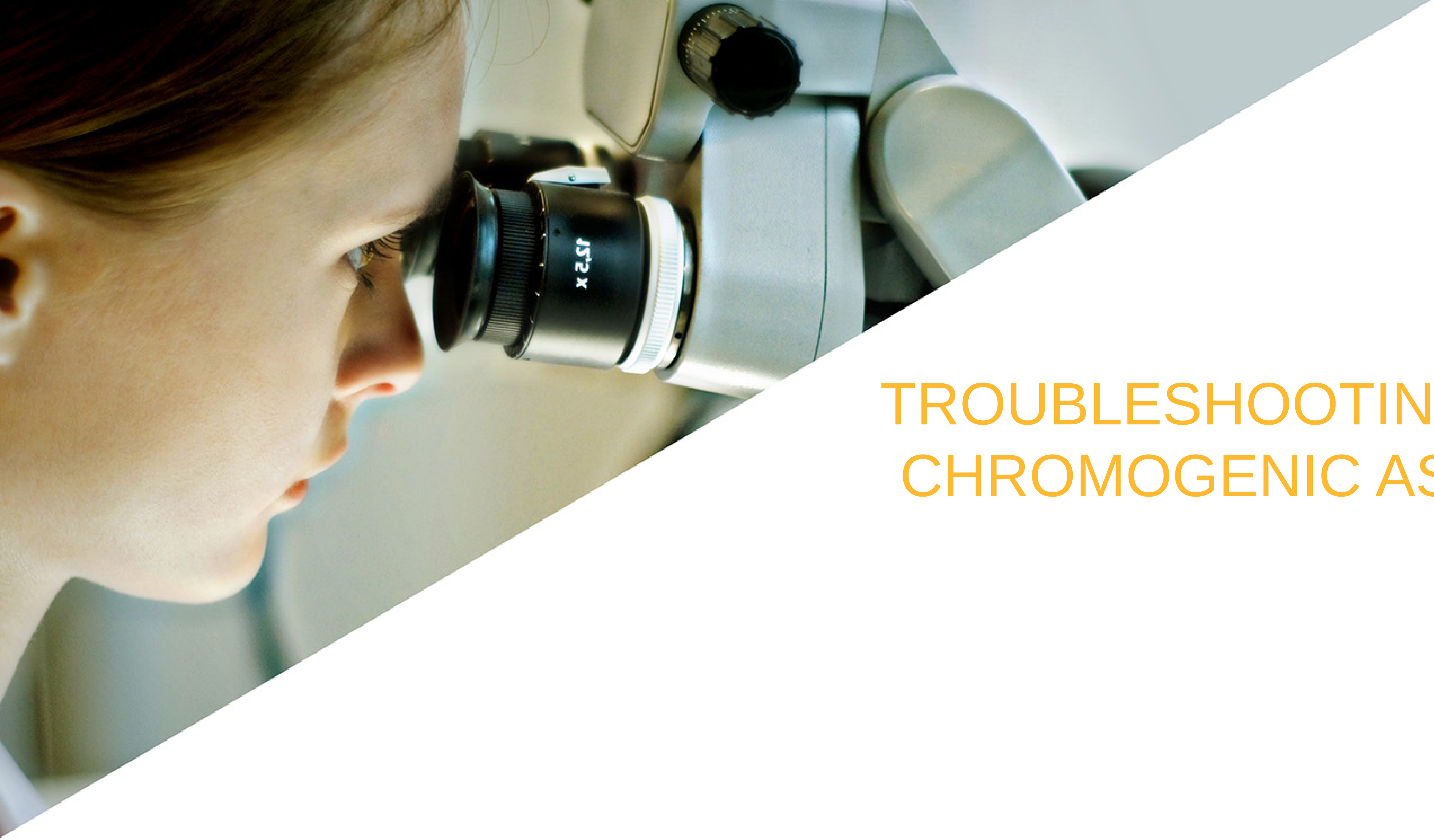
EXAMPLE OF SUCCESFUL RNASCOPE ® RESULTS

Hs POLR2A/Alexa 488 | **Hs PPIB/Atto 550** | **Hs UBC/Atto 647** | **Merged**



RNAscope Multiplex Fluorescent Assay
Amp 4 ALT A

Human Hela Cell Line



TROUBLESHOOTING TIPS CHROMOGENIC ASSAYS

FACTORS AFFECTING RNASCOPE® ASSAY PERFORMANCE

☒	Fixation conditions are not optimal
☒	RNA is degraded
☒	Hybridization conditions not optimal
☒	Samples drying during assay

THE SOLUTIONS

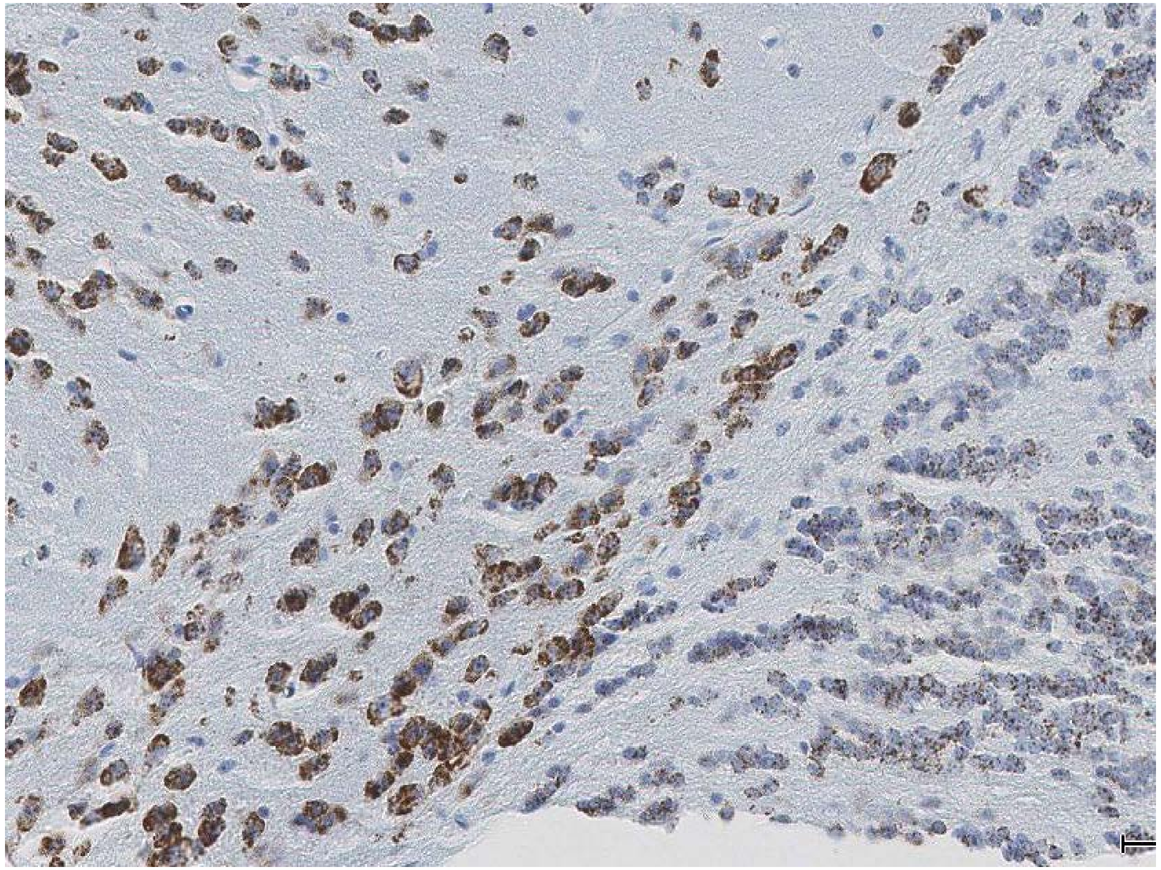
☑	Fix samples as recommended. E.g., for FFPE use 10% NBF RT, 16-32 hrs
☑	Acquire new samples and assess RNA quality
☑	Use the HybEZ hybridization oven only
☑	Use Immedge pen and add adequate reagents to avoid drying

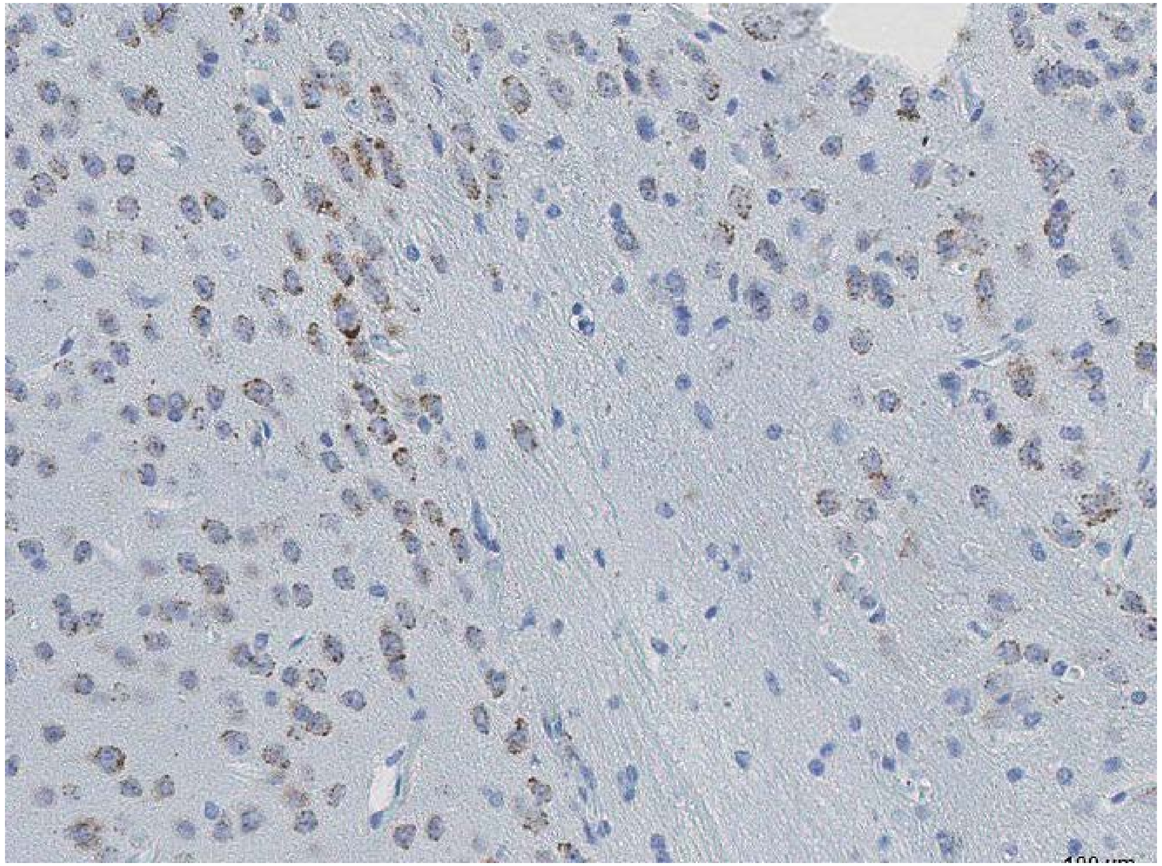
NBF: Neutral Buffered Formalin

IMPACT OF FIXATION CONDITIONS

24 hours fixation/**Optimal**

3 weeks fixation/**Over fixed** Synaptophysin





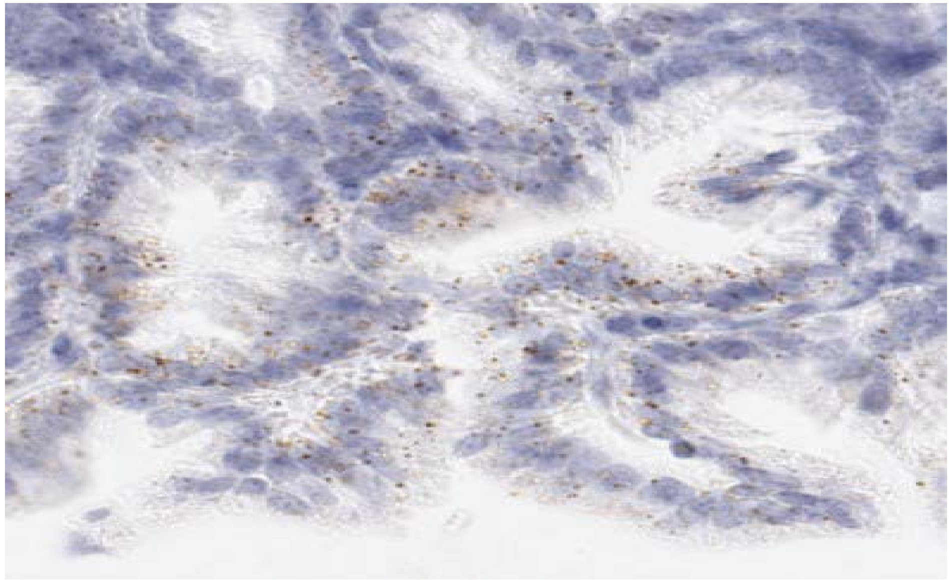
Sample: FFPE brain sample

Assay: RNAscope 2.0 Brown

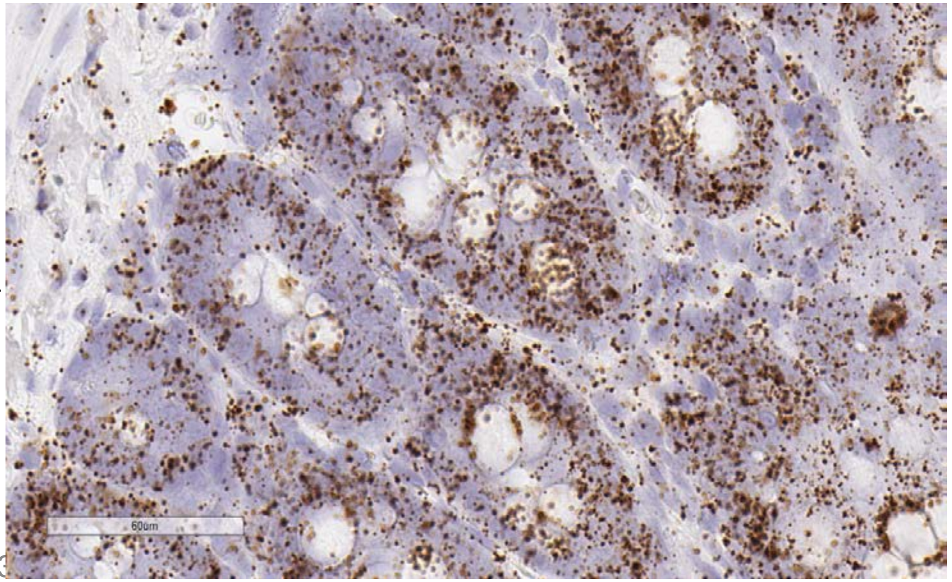
TIP: Sample fixation has a great effect on the success of your assay
Solution: Increase pretreatment for better target accessibility

TROUBLESHOOTING: **UNDER FIXATION**

Positive control, Rn PPIB



Positive control, Rn PPIB



Sample: Flash Frozen followed by FFPE sample preparation, Rat intestines

Assay: RNAscope 2.0 HD Brown

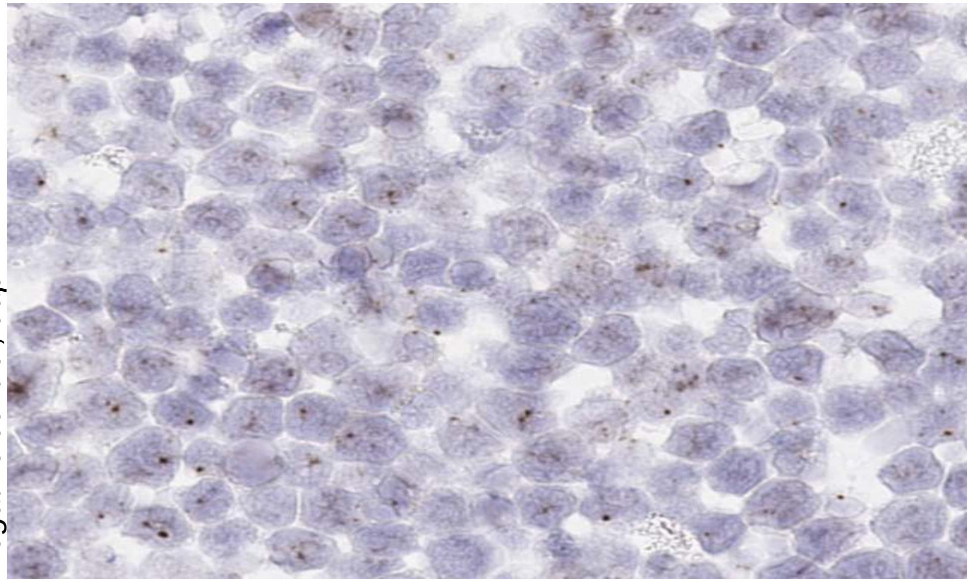
Issue: Weak staining, destroyed morphology, fresh frozen sample is under fixed

Optimization: Fixation according to recommended guidelines for FFPE samples

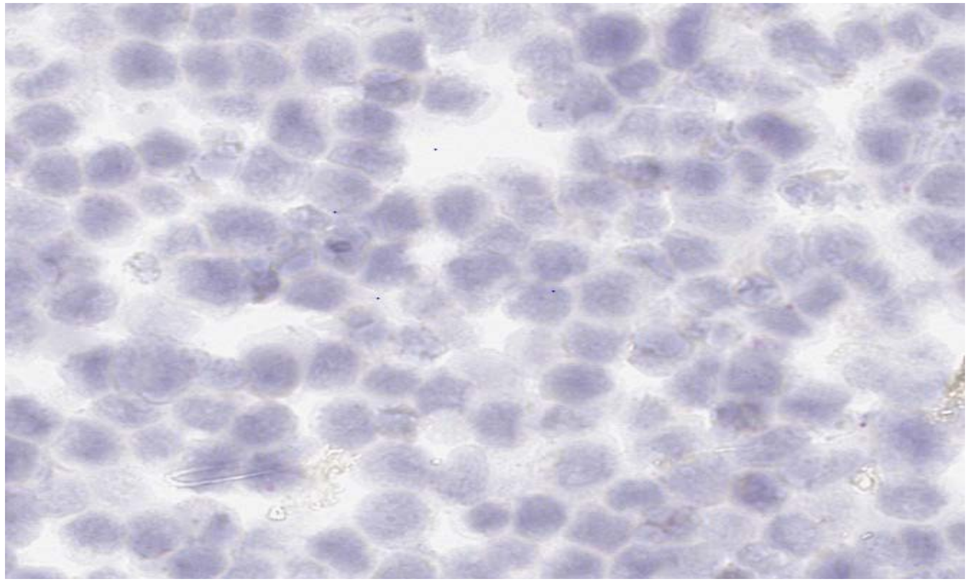
Result: Strong staining for positive control, PPIB, intact morphology

TIP : Refer to the Troubleshooting Guide
http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/

TROUBLESHOOTING: **ASSAY WORKFLOW**



Negative control, dapB



Negative control, dapB

Sample: FFPE Hela pellet

Assay: RNAscope 2.0 HD Brown

Issue: Tissue dried out, high background

Optimization: Do not allow drying between amplification steps. Use the Immedge hydrophobic barrier pen

Result: Clean background

TIP: Refer to the Troubleshooting Guide; http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/

REFER TO SAMPLE PRETREATMENT GUIDELINE

Tissue Pretreatment Guidelines

Follow the recommended pretreatment conditions based on your tissue type for:

- Any new or previously untested FFPE tissues
- Samples prepared suboptimally

Guidelines for Optimal Tissue Pretreatment

- ❑ Test representative samples with positive and negative control probes. [Controls should be: Positive = uniform signal; negative = blank].
- ❑ Fix sample in **FRESH 10% NBF for 16–32 HOURS at ROOM TEMPERATURE.**

  NOTE: Do not fix at 4°C. DO NOT fix for < 16 hrs or >32 hrs. Refer to Table 1 for under/over-fixed tissue pretreatment guidelines.
- ❑ Vary **PRETREAT 2** and/or **PRETREAT 3 TIME** based on your tissue type (see Table 2).

  NOTE: Certain Xenografts and Cell Pellets, require very mild pretreatment (**PRETREAT 2** for 8 min, **PRETREAT 3** for 15 min).

Table 1. Tissue Pretreatment Guidelines

Reagent	Mild	Standard	Extended
Pretreat 2	15 min	15 min	30 min
Pretreat 3	15 min	30 min	30 min

Table 2. Tissue Pretreatment Table

Species	Tissue type	Pathology	Pretreat Condition
Mouse / Rat	Intestine	Normal	Standard
	Intestine	Tumor	Standard
	Embryo	Normal	Standard
	Brain	Normal	Standard
	Spleen	Normal	Mild
	Eye/Retina	Normal	Standard
	Liver	Normal	Extended
	Kidney	Normal	Standard
Human	Breast	Tumor	Standard
	Colon	Tumor	Standard
	Colon	Normal	Standard
	Lung	Tumor	Standard
	Lung	Normal	Standard
	Prostate	Tumor	Standard
	Prostate	Normal	Standard
	Lymph node	Tumor	Mild
	Lymph node	Normal	Mild
	Tonsil	Normal	Mild
	Pancreas	Normal	Standard
	Cervical	Cancer	Standard

Species	Tissue type	Pathology	Pretreat Condition
Human	Cervical	Normal	Standard
	Cervical dysplasia	Abnormal	Standard
	Brain	Tumor	Standard
	Brain	Normal	Standard
	Head	Cancer	Standard
	Neck	Cancer	Standard
	Liver	Cancer	Standard
	Kidney	Normal	Standard
	Skin	Normal	Standard
	Melanoma	Tumor	Standard
	Nevus	Benign	Standard
	Placenta	Normal	Standard
	Skin (TMA*)	Normal	Standard
	Breast (TMA)	Normal	Standard
	Melanoma (TMA)	Normal	Standard
	Nevus (TMA)	Benign	Standard
	Stomach (TMA)	Normal	Standard
	Stomach (TMA)	Tumor	Standard
	Cell pellets**	—	Mild
	HeLa cells† (ACD control)	—	Standard

* Tissue Microarray
** Fixed with 10% NBF
† Fixed with 10% Formaldehyde/PBS

For information about species or tissue type not listed here, contact support at support@acdbio.com.

TIP : Refer to the user manual for tissue specific pretreatment guidelines

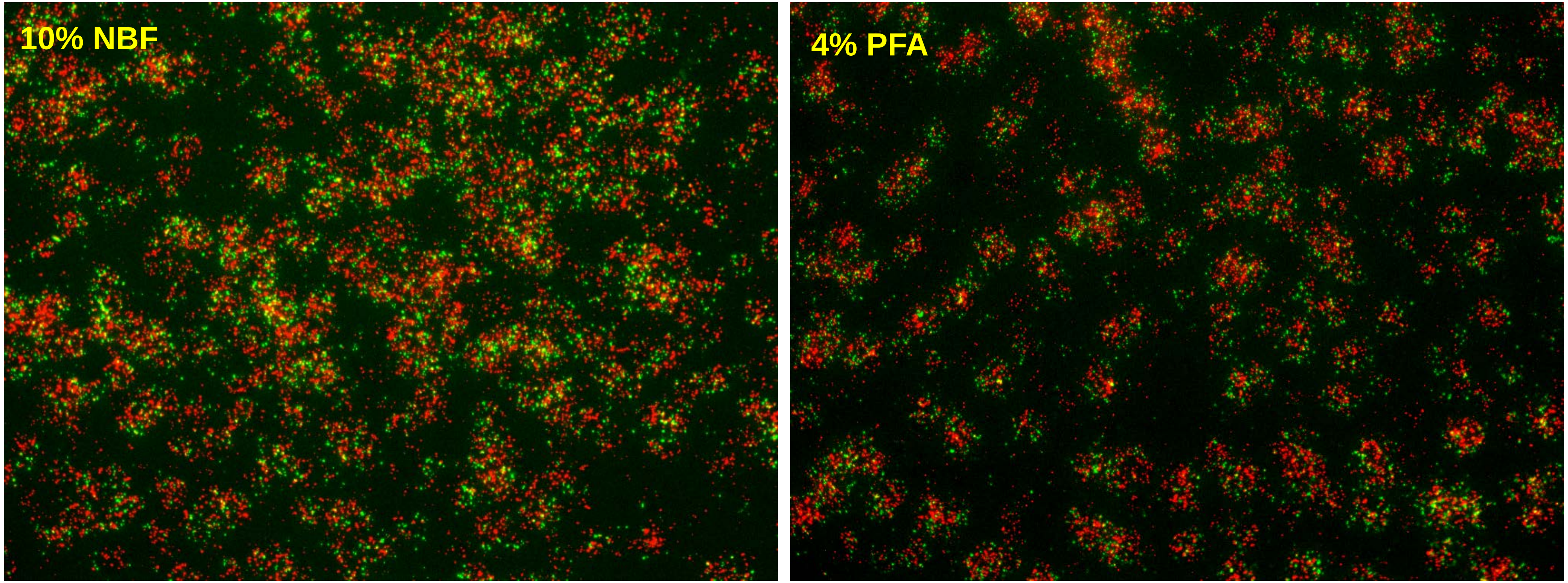


TROUBLESHOOTING TIPS MULTIPLEX FLUORESCENT ASSAY

IMPACT OF FIXATION CONDITIONS

2-plex Mouse Positive Control Probe Mm POLR2A/PPIB

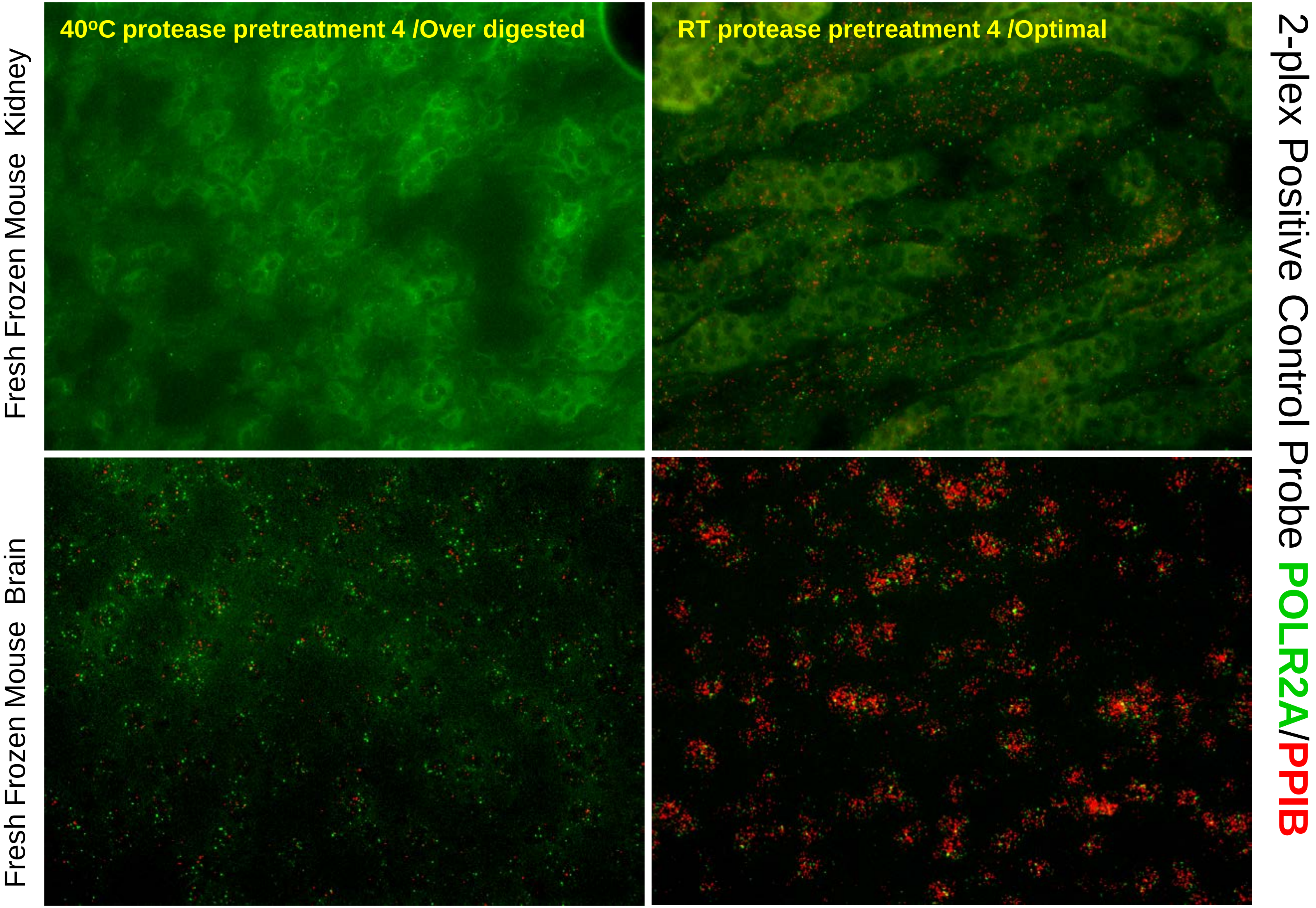
Fresh Frozen Mouse Brain



Experiment condition: 10% NBF, 15 min Fixation, Pretreatment 4, RT

TIP: Sample fixation can have a great effect on the success of your assay
Solution: Use prechilled 10% NBF at 4ºC

IMPACT OF TEMPERATURE ON PRETREATMENT CONDITIONS

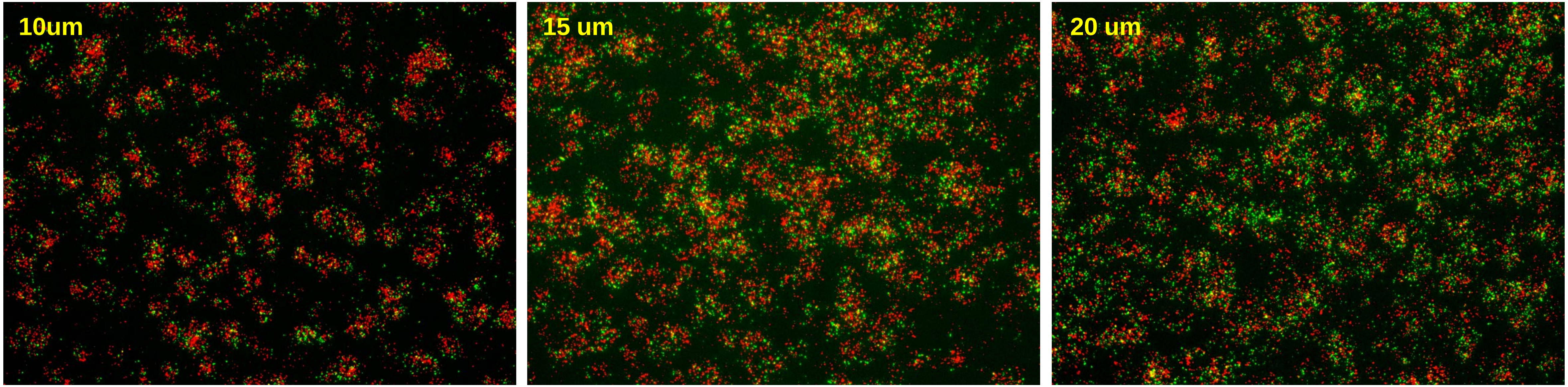


TIP: Pretreatment temperature has a great effect on the success of your assay
Solution: Perform pretreatment at RT to avoid over digestion of your sample

IMPACT OF SAMPLE THICKNESS

2-plex Mouse Positive Control Probe Mm POLR2A/PPIB

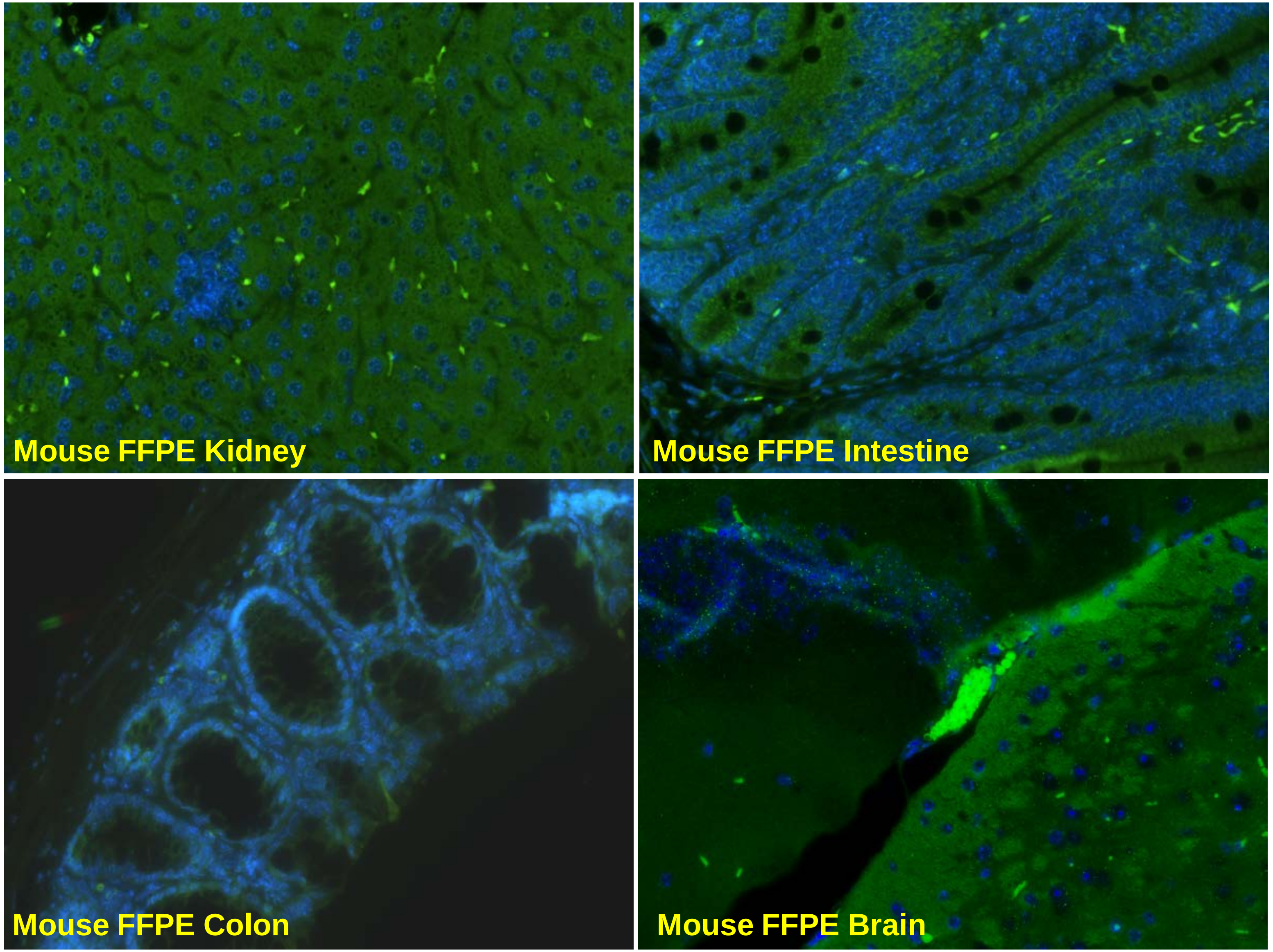
Fresh Frozen Mouse Brain



Experiment condition: 10% NBF, 15 min Fixation, Pretreatment 4, RT

TIP: Sample thickness can signal in your samples
Solution: Use recommended sample thickness, 10-20um

AUTOFLUORESCENCE



TIP: FFPE sample have inherent autofluorescence

Solution: Use appropriate background correction software to reduce autofluorescence

MULTIPLEX FLUORESCENT ASSAY 101—PROBLEMS AND SOLUTIONS

SOURCE	ISSUE	PROBLEM	SOLUTION
Microscopy	No/weak signal Nonspecific signal	1. Wrong filter setting/longer emission cut off 2. Wrong exposure 3. Inappropriate imaging enhancing with software	1. Use correct filter settings 2. Do not use using autoexposure at first, verify signal with naked eye 3. Use known image enhancing software e.g. Nuance
Sample	No/weak signal	1. Compromised RNA quality 2. Sample preparation (high autofluorescence background on the sample	1. Use new sample with proven RNA quality 2. Follow the pretreatment guideline recommended 3. Always perform assay with 3-plex positive control and 3-plex negative probes to assess RNA quality 4. Always check signal with naked eye under objective lens first

MULTIPLEX FLUORESCENT ASSAY 101—TIPS AND TRICKS

- Be aware of the suggested filter settings for your microscope
- Use the suggested pretreatment condition
- Use the sample preparation protocol (PART 1) for your samples for optimal results
- Always run a 3-plex positive control and negative control to assess RNA quality and to verify microscope setting are appropriate
- Always evaluate the results by eye first before capturing images



FREQUENTLY ASKED QUESTIONS

FREQUENTLY ASKED QUESTIONS

- RNAscope assay compatibility with different tissues

  RNAscope manual assay can be used with FFPE, fresh-frozen, fixed-frozen and cultured cells. RNAscope automated assays are primarily supported with the FFPE tissue.
  Please refer to the User Manual Selection Guide: http://www.acdbio.com/technical-support/downloads

- Key differences between RNAscope ISH assay and IHC

  No cooling is required during Epitope retrieval, users should directly put the slides in water at room temperature, dehydrate and proceed to Pretreatment 3 step as per the manual Part 1

TIP: Visit www.acdbio.com/support for additional FAQs

GUIDELINES TO FOLLOW WITH RNASCOPE® ASSAY

- ✓ Read the manual and perform RNAscope exactly
- ✓ Utilize optional stopping points
- ✓ Flicking/tapping the slides for adequate drying of slides
- ✓ Storage in desiccant (FFPE) for RNA integrity
- ✓ Always use fresh reagents
- ✓ Warm probes and wash buffer at 40°C, precipitation occurs during storage
- ✓ Remember to optimize pretreatment conditions, when you switch tissues

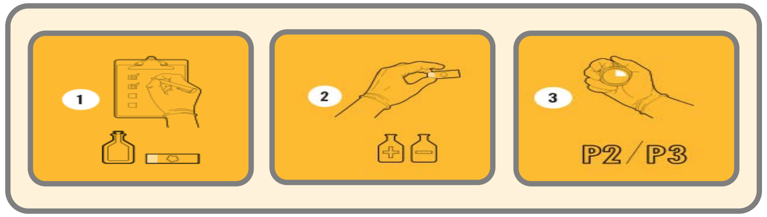
TIP: Refer to the Troubleshooting Guide
http://www.acdbio.com/technical-support/downloads/rnascope-ish-guide-troubleshooting/

SUMMARY

1. Reviewed RNAscope® technology

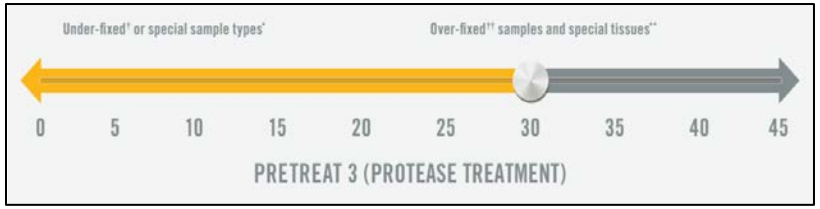
   - ZZ probe design and the workflow for manual assays

2. Tips for getting started with RNAscope® in your lab
   – 3 easy steps to getting started in your lab



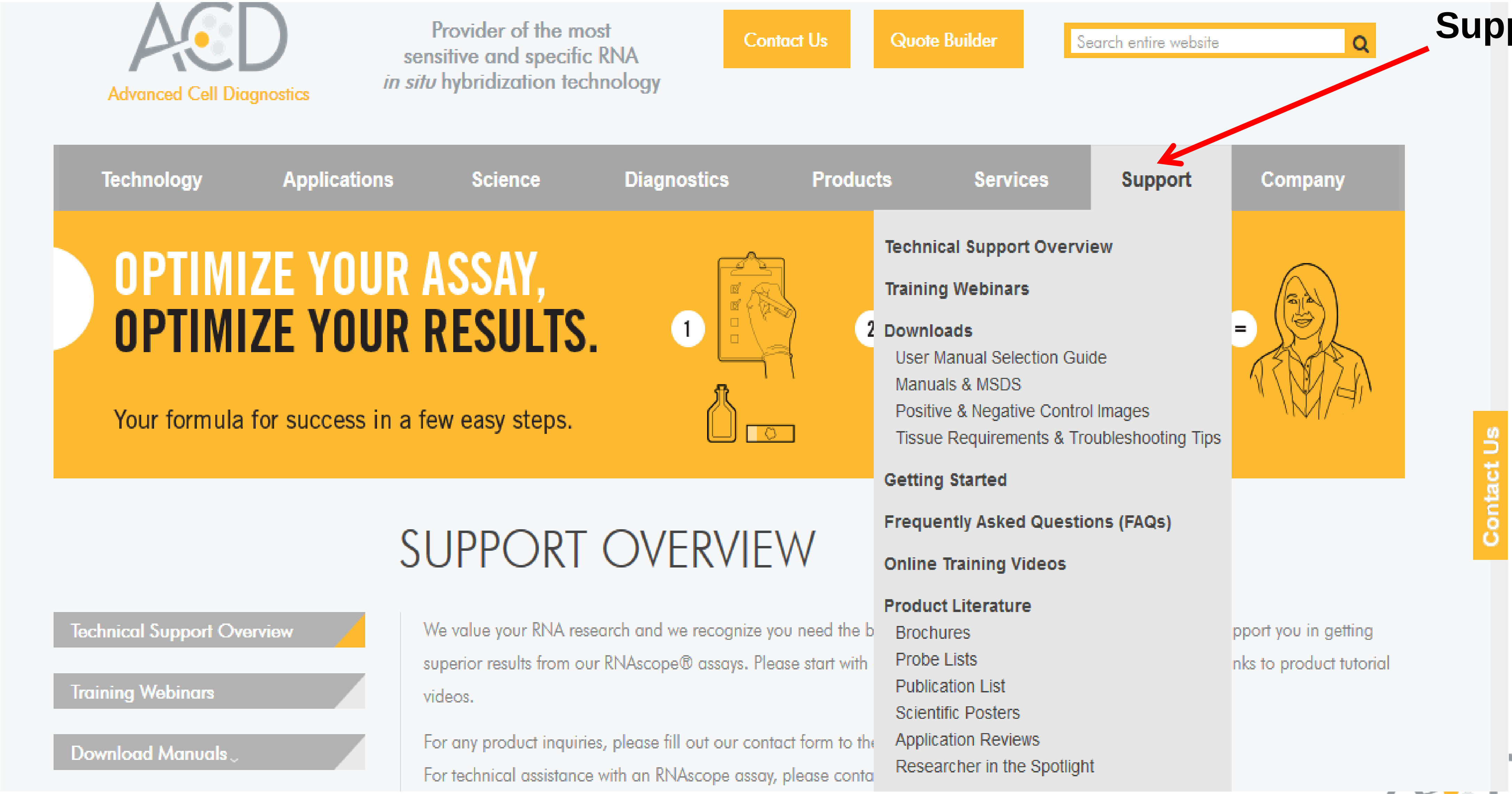
3. Tips for success with RNAscope® assay, when you switch tissues
   – Pretreatment 2 and 3 optimization





VISIT THE SUPPORT PAGE TO LEARN MORE

Support tab



TIP: Visit www.acdbio.com/technical-support/support-overview

CONTACT ACD SUPPORT

- Support via email –support@acdbio.com
- Support via phone-1-877-376-3636, option 3
  - Time 8:00am-6:00pm PST
- Support Resources available on website www.acdbio.com



Manuals	Getting Started	FAQs	Videos	Product Literature
Download manuals, technical notes and MSDS.	Simple tips & tricks for you to get the best RNAscope result from day1.	Browse through our product frequently asked questions or add one of your own.	View our product and workflow videos on our Video page.	Find RNAscope publication lists, gene lists and download product brochures.
Go →	Go →	Go →	Go →	Go →

QUESTIONS?



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